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The HOT Study: the development, management and results from a phase IIB, randomised controlled trial of heme arginate in recipients of deceased donor renal transplants.

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Declaration

I, Rachel Alexandra Barclay Thomas, declare that this thesis was composed myself and the work contained herein is my own and was done by myself with the technical input from people I have acknowledged in the appropriate section.

This work has not been submitted for any other degree or professional qualification.

Rachel A B Thomas

Abstract

Aims

There are few proven therapies that can protect against the inevitable ischaemia reperfusion injury (IRI) that occurs during renal transplantation. IRI increases the likelihood of delayed graft function (DGF), which negatively impacts on the long-term survival of a transplanted kidney. One enzyme of interest, heme oxygenase-1 (HO-1), degrades heme and protects against the oxidative stress that occurs secondary to IRI. Clinical renal recipients with higher HO-1 levels have improved graft function post transplant. Heme arginate (HA), a form of hemin, which has been used to treat porphyria for over 30 years, has repeatedly been shown to induce HO-1 in *in vivo* and *in vitro* macrophages. It is one of the few HO-1 inducers approved for clinical use and healthy volunteer studies confirmed that HA could also safely induce HO-1 in humans.

Prior to the formal start of the MD, the University of Edinburgh successfully applied to NHS Blood and Transplant for funding to investigate whether giving HA to recipients of deceased donor renal grafts prior to transplant could upregulate HO-1 and whether this had any effect on the function and health of the grafts. This MD aims to explain the background behind the proposed study, the process of study approval, planning and trial logistics and protocol. This thesis then describes the methods of sample analysis, the results and future directions for the HOT (Heme Oxygenase-1 in renal Transplantation) study.

Methods

The HOT study planning and approval process took eight months and the first participant was randomised in January 2012. The study was sponsored by ACCORD, a joint company from University of Edinburgh and NHS Lothian, and recruited patients from the Edinburgh Royal Infirmary Transplant Unit. The protocol was followed to ensure that 40 recipients were randomised blind to either active (two doses 3mg kg^{-1} HA: pre-operatively, day 2) or placebo (NaCl: same schedule).

To ensure that the primary outcome was fulfilled, recipient blood was taken daily for peripheral blood mononuclear cells (PBMC) extraction. After further blinding steps, the PBMCs were analysed for HO-1 protein and mRNA. The secondary outcome measures involved collecting urine for analysis of urinary biomarkers (KIM-1 and NGAL), taking renal graft biopsies pre-op and day 5 for renal HO-1 analysis and collecting renal function data. DGF was calculated daily. To ensure that all adverse event data was captured, the recipients were closely reviewed for 7 days and their renal function was monitored for 90 days.

Results

The final participant was recruited in May 2013 within the predicted timescale and to budget. This participant completed follow-up in August 2013. Of the 40 participants, three received the infusion but did not receive a transplant and therefore could not give primary outcome data. The remaining 37 did and this was analysed. Adverse events were equivalent between groups and there were no adverse reactions to HA.

HA upregulated PBMC HO-1 protein at 24 hours compared to placebo: HA 11.1ng/ml (1.0- 37.0) vs. placebo 0.14ng/ml (-0.7- 0.3)(p=<0.0001). PBMC HO-1 mRNA was also increased: HA 2.73 fold (1.8- 3.2) vs. placebo 1.41 fold (1.2- 2.2) (p=0.02).

HA increased HO-1 protein immunopositivity in day 5 renal tissue compared with placebo: HA 0.21 (-24- 0.7) vs. placebo -0.03 (-76- 0.15) (p=0.02) and the percentage of HO-1 positive renal macrophages also increased: HA 50.8 cells per HPF (40.0- 59.8) vs. placebo 22.3 (0- 34.8) (p=0.012). Renal HO-1 mRNA was also increased in HA group: 2.02 (0.20- 4.03) fold increase compared to 1.68 (0.75- 10.39) fold in the placebo group but it was not significant (p= 0.451).

Urinary biomarkers were reduced after HA but not significantly so.
Histological injury and DGF rates were similar between the groups.

Conclusion

HA is safe and effective in renal transplant recipients as reported in this phase II, randomised, placebo controlled, blinded, single-centre study. The primary outcome was achieved and demonstrated for the first time that HA induces HO-1 in peripheral and renal macrophages in kidney transplant recipients.

There was also evidence that HA increased HO-1 expression in renal tissue. There was no evidence that HA improved renal function or reduced injury as seen in animal models but it is recognised that the sample size was small and the study was not powered to these endpoints.

Larger studies are planned to determine the impact of HO-1 upregulation on clinical outcomes and evaluate the benefit to patients at risk of IRI. The plans for HOT2 are expanded in this thesis.

Lay Summary

When a kidney is transplanted from one patient to another, there is an inevitable time period when the organ does not receive blood and is therefore deprived of oxygen. This results in a serious disease called ischaemia reperfusion injury (IRI) of which there is no cure. The more IRI the kidney suffers, the worse it performs when it is transplanted into a recipient. This MD investigated a way to protect kidneys against IRI with the goal of offering hope to patients who are at risk of this disease in transplantation and, also potentially in other illnesses where a lack of blood flow is an issue, such as heart attacks and strokes.

Heme-oxygenase-1 (HO-1) is present naturally in nearly all of our cells. It is an enzyme that breaks down harmful waste products into positive protective ones. This MD describes the HOT study (Heme Oxygenase-1 in renal Transplantation), which used Heme arginate (HA) to try to increase the amount of HO-1 present in recipients of kidneys from deceased donors. Previous research has shown that people with higher HO-1 levels do better after transplantation and that HA can increase HO-1 in animals and healthy volunteers.

40 recipients who were admitted for a potential kidney transplant volunteered and were randomised to receive either HA or a saline solution, known as placebo. Once the transplant had been performed, blood and urine was taken daily for analysis. A sample of the donor kidney was taken before and 5 days after transplant. The trial was completed on time and to budget.

The HOT study achieved its primary aim and showed that HA was safe. It was also effective because it increased the amount of HO-1 in the patient's blood and kidney tissue. The kidneys appeared to be healthier and work better after HA but this was not as obvious because the number treated was small.

This is exciting news because there are so few other options available to improve outcomes in transplantation. The number of people who need kidneys exceeds the limited resource and HA may offer a chance to improve the health of kidneys after transplant, thereby maximising our supply. Larger studies are planned to see if HA really can improve the function of kidneys post-transplant and also to expand into other areas of disease.

Dedication

This MD was only possible because of the support and unconditional love from three wonderful people in my life, Richard, Grayson and Penrose. I owe you all so much and will be repaying you with lie-ins, chocolate and LEGO forever.

Acknowledgements

I am indebted to my MD supervisors David Kluth and Lorna Marson. They provided infinite support and advice and there would be no MD without them. I hope this is the start of a lifetime of collaborations.

The members of the Centre for Inflammation Research PIG labs provided advice, techniques and friendship and I am so grateful. Special thanks to Katherine and Emily. Thanks also to David Ferenbach for the background information and statistics.

I owe much to my parents for their support throughout my education and career. Their constant encouragement helped me complete this MD and their joy at the associated Moynihan Medal Prize will stay with me forever.

The HOT study was only possible thanks to the generous funding from NHS Blood and Transplant and help from the laboratory staff at QMRI and staff in the Radiology department, Pathology department and the Transplant Unit in Royal Infirmary of Edinburgh. The East of Scotland recipient transplant coordinators were crucial to the study and my grateful thanks to them. Cat Graham and Sharon Harden at University of Edinburgh provided expert statistical support.

Most of all thank you to the participants who participated in the HOT study. Without them there would have been no trial and therefore no possibility to improve the situation for those on the transplant waiting list.

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Abbreviations

ACCORD	Academic and Clinical Central Office for Research and Development
AE	Adverse event
AP-1	Activator protein-1
AR	Adverse reaction
ASA	American Society of Anaesthesiologists (classification)
ATF-2	Activating transcription factor 2
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
AV	Arterio-venous
AUC	Area under the curve
BSA	Bovine serum albumin
cGMP	Cyclic guanosine monophosphate
CI	Chief Investigator
CIT	Cold ischemic time
CO	Carbon monoxide
CONSORT	Consolidated Standards of Reporting Trials
CoPP	Cobalt-protoporphyrin
CORM	CO–releasing molecules
CRP	C-reactive protein
CTA	Clinical trial authorisation
CTIMP	Clinical trial of investigational medicinal product
DBD	Donor after brain death
DCD	Donor after circulatory death
DCF	Data collection forms
DGF	Delayed graft function
DMC	Data monitoring committee
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECD	Extended criteria donors
ECL	Electrochemiluminescence

ECTU	Edinburgh Clinical Trials Unit
eGFR	(estimated) Glomerular Filtration Rate
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
FACS	Fluorescence-activated cell sorting
FBC	Full blood count
FCS	Fetal calf serum
GCP	Good clinical practice
GLP	Good laboratory practice
GP	General Practioner
GT	Guanosine thymine
HA	Heme arginate
HLA	Human leucocyte antigen
HO-1	Heme oxygenase-1
HIF	Hypoxia inducible factor
HPF	High powered field
HRP	Horse-radish peroxidase
IHC	Immunohistochemistry
IF	Immunofluorescence
IFN	Interferon
IL-	Interleukin (1β, 2, 6, 10, 12, 18)
IMP	Investigational medicinal product
IQR	Interquartile range
IPC	Ischaemic preconditioning
IRAS	Integrated research application system
IRI	Ischaemia reperfusion injury
ISF	Investigator site file
ITU	Intensive therapy unit
KIM-1	Kidney injury marker 1
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection

MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare products Regulatory Agency
MMF	Mycophenolate mofetil
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NGAL	Neutrophil gelatinase-associated lipocalin
NKT	Natural killer T-cells
NHS	National Health Service
NO	Nitric oxide
NF-κB	Nuclear factor κB
OSOM	Outer stripe of medulla
QA	Quality assurance
QMRI	Queen's Medical Research Centre
qRT-PCR	Real time reverse transcription polymerase chain reaction
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Principle investigator
PRP	Platelet rich plasma
PSGL	P-selectin glycoprotein ligand
RCT	Randomised controlled trial
REC	Regional Ethics Committee
R&D	Research and Development
RDS	Research and development statistics
RFU	Relative fluorescent units
RIE	Royal Infirmary of Edinburgh
RIPA	Radioimmunoprecipitation assay
RIPC	Remote ischaemic preconditioning
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SAE	Serious adverse event
SAR	Serious adverse reaction
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SmPC	Summary of product characteristics
SIRS	Systemic inflammatory response syndrome
Sp1	Specialty protein 1
SURF	Shared University Research Facility
SUSAR	Suspected unexpected serious adverse reaction
TBS	Tris- buffered saline
TBST	Tris-buffered saline and Tween
TGF- β1	Transforming growth factor- β1
TNF-α	Tumour necrosis factor- α
TMB	Tetramethylbenzidine
TSC	Trial steering committee
U&Es	Urea and electrolytes
UAR	Unexpected adverse reaction
UBCR	Urinary biomarkers: creatinine ratio
USF	Upstream stimulatory factor
WIT	Warm ischaemic time
WTCRF	Welcome Trust Clinical Research Facility

Chapter 1.

Introduction

1.1 Kidney transplantation

1.1.1 *Background*

The optimal treatment for the majority of patients with end stage renal disease (ESRD) is kidney transplantation. Despite the risks of surgery and subsequent graft problems, life expectancy is improved after cadaveric renal transplantation compared to life remaining on dialysis, both nationally (Oniscu, Brown et al. 2005) and internationally (Wolfe, Ashby et al. 1999). Renal transplantation is also cost-effective with a positive impact on quality of life (Fiebiger, Mitterbauer et al. 2004).

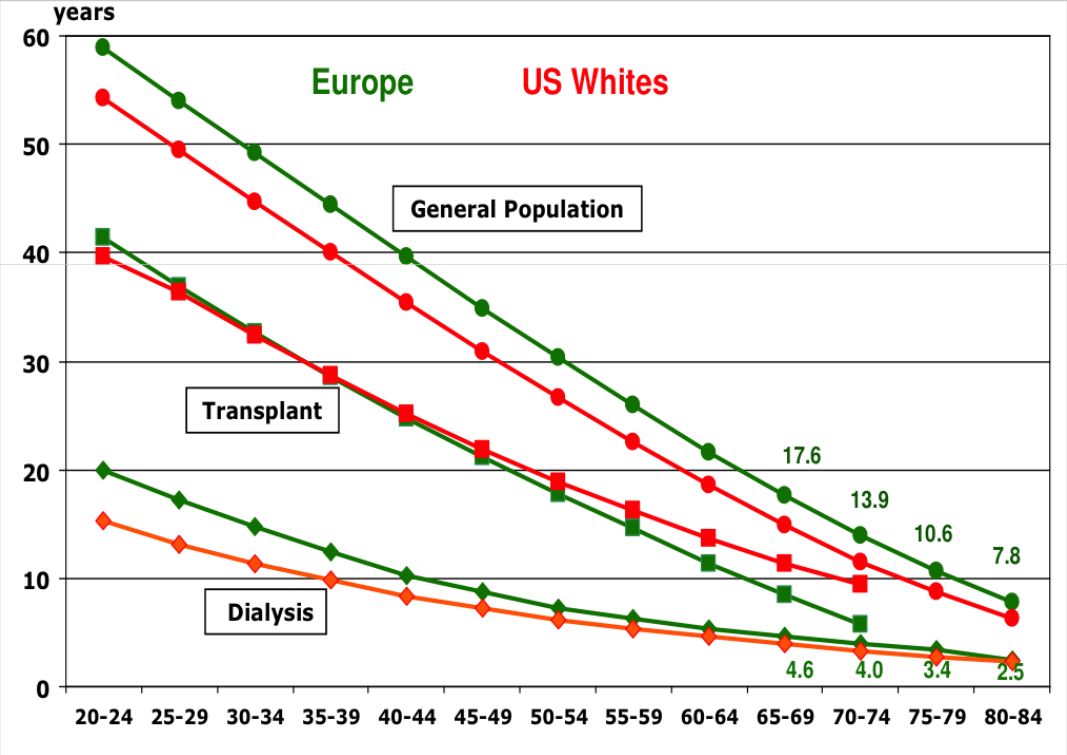


Figure 1-1 Comparison between life expectancies of general population, patients after transplant and dialysis patients (from Dr David Ferenbach, using US RDS data)

Organs from living donors demonstrate consistently superior results compared to organs from deceased donors regardless of the degree of HLA match (Pratschke, Wilhelm et al. 2001). Poor kidney function after transplant, known as delayed graft function (DGF), complicates between 5-50% of cadaveric renal transplants, and is around 50% more common than after living donor transplants (Quiroga, McShane et al. 2006). The inevitable difference in cold ischaemic time (CIT) between cadaveric and living donor grafts influences outcome as each additional hour of CIT increases the risk of graft failure (Debout, Foucher et al. 2015). DGF is associated with prolonged hospitalisation and increased risk of acute rejection. It also has an impact on long-term sequelae including reduced graft survival and acute rejection with financial implications (Quiroga, McShane et al. 2006, Yarlagadda, Coca et al. 2009).

Unfortunately, most patients with ESRD do not have a living donor available to them and the only alternative is cadaveric donation. However, demand for kidneys outstrips supply and as a result, the criterion of acceptable donor organs has been extended to include organs that previously would have been deemed unsuitable for donation. These include organs from older donors and organs donated after circulatory death (DCD). The principal difference between DCD and donation after brain death (DBD) is the period of warm ischaemia to which the kidney is exposed, which is greater in DCD organs. While DCD organs are more vulnerable to injury from prolonged ischaemia (Tullius, Reutzel-Selke et al. 2000), DBD organs are not risk-free because brain death itself causes rapid and massive up-regulation of a variety of pro-inflammatory mediators and acute phase proteins in the prospective organ donor (Floerchinger, Oberhuber et al. 2012). In an attempt to reduce the

ischaemic time of DCD organs, allocation is weighted in favour of shorter travel times. Despite this concern, DCD and DBD kidneys have similar outcomes and DCD organs are no longer considered contentious (Doshi and Hunsicker 2007).

1.1.2 *Extended criteria donors (ECD)*

The definition of extended criteria donors include donors over 60 years old and donors over 50 years old with two of the following; hypertension, cerebrovascular accident as cause of death, or pre-retrieval serum creatinine of >15mg/dl (Audard, Matignon et al. 2008).

Kidneys from ECD merit transplantation because survival of recipients of such grafts is still better than dialysis patients waiting for a standard organ (Ojo, Hanson et al. 2001, Snoeijs, Schaubel et al. 2010). There is on-going debate about who benefits most from ECD organs and whether allocation of these organs should be modified as in the Eurotransplant Senior Programme. Across Austria, Belgium, Croatia, Germany, Hungary, Luxemburg, the Netherlands and Slovenia organs from deceased donors over 65 years old are only allocated to recipients older than 65 years without the use of donor HLA typing. The three year graft outcomes from those who received an age-matched organ were as good as HLA matched transplants (Cohen, Smits et al. 2005). Subgroup analysis shows that the most appropriate people to receive ECD kidneys are patients over 60 years old on dialysis or younger diabetics (Gaston, Danovitch et al. 2003). Although ECD kidneys are associated with significantly more complications, including increased risk of DGF, Fraser et al demonstrated no significant difference in five-year survival compared to standard

criteria donors. As a result, ECD organs will continue to form an increasing proportion of all transplants but research into ways to reduce their associated shortcomings is required (Fraser, Rajasundaram et al. 2010).

With advances in immunosuppression, acute rejection is now rare and the most pressing dilemma in transplantation is how to prolong the lifespan of each organ in order to reduce the numbers of repeat transplants and therefore, decrease the rate of relisting and dialysis burden.

1.2 Ischaemia reperfusion injury in transplantation

Although donor characteristics are an important predictor of graft function, perioperative factors also play a role. During transplantation the kidney inevitably suffers a temporary interruption and then resumption in its blood supply. This phenomenon is known as ischaemia-reperfusion injury (IRI) and is a significant alloantigen-independent cause of DGF.

The pathogenesis of IRI is not fully understood but is likely to represent a network of interactions facilitated by oxidative molecules and inflammatory cell mediators where activated leucocytes, endothelium and platelets are linked in a vicious circle (Devarajan 2006, Kinsey, Li et al. 2008). The release of vasoconstrictors and formation of platelet microthrombi disrupt the microvasculature of the kidney, which results in further injury (Furuichi, Gao et al. 2006, Basile 2007, Holzen, August et al. 2008, Kinsey, Li et al. 2008, Kinderlerer, Pombo Gregoire et al. 2009).

Prolonged **ischaemia** leads to cellular necrosis. In the presence of hypoxia, the ATP pump fails, allowing calcium, sodium and water to enter the cells. ATP is broken down into the potentially toxic hypoxanthine, which accumulates because it cannot be catalysed in the anoxic environment. Endothelial injury leads to a cascade of events including leucocyte margination and extravasation, increased expression of inflammatory cytokines (IL-1β, IL-2, IL-6 and TNFα) and smooth muscle vasoconstriction. Hypoxia itself directly activates many of the pathways common to both ischaemia and inflammation (Eltzschig and Collard 2004).

When the kidney is **reperfused** with oxygen, the endothelium is reactivated and the damaging cycle is amplified, rather than alleviated (Koo and Fuggle 2000). Although restoration of the blood flow is essential to prevent permanent damage to the organ, reperfusion causes more damage than ischaemia alone. Liver transplant biopsies have more severe histological changes after three hours of ischaemia and one hour of reperfusion than ischaemia alone for the same period (Varadarajan, Golden-Mason et al. 2004). When oxygen is reintroduced, the excess hypoxanthine is converted to reactive oxygen species (ROS), which causes further tissue injury via lipid peroxidation, leucocyte activation, expression of MHC molecules, recruitment of antigen presenting cells and complement activation (Eltzschig and Collard 2004).

In addition to this inevitable IRI damage, there is also a risk of the no-reflow phenomenon in any transplanted organ. This is said to occur when the blood supply to an ischaemic organ is re-established but microvasculature blood flow remains disrupted. On-going endothelial cell disruption, platelet and leucocyte accumulation and aggregation is thought to be responsible (Brodsky, Yamamoto et al. 2002). In renal transplantation, the end result is a higher incidence of DGF and a risk of graft failure. DGF is described in discussion section 4.1.5.1.

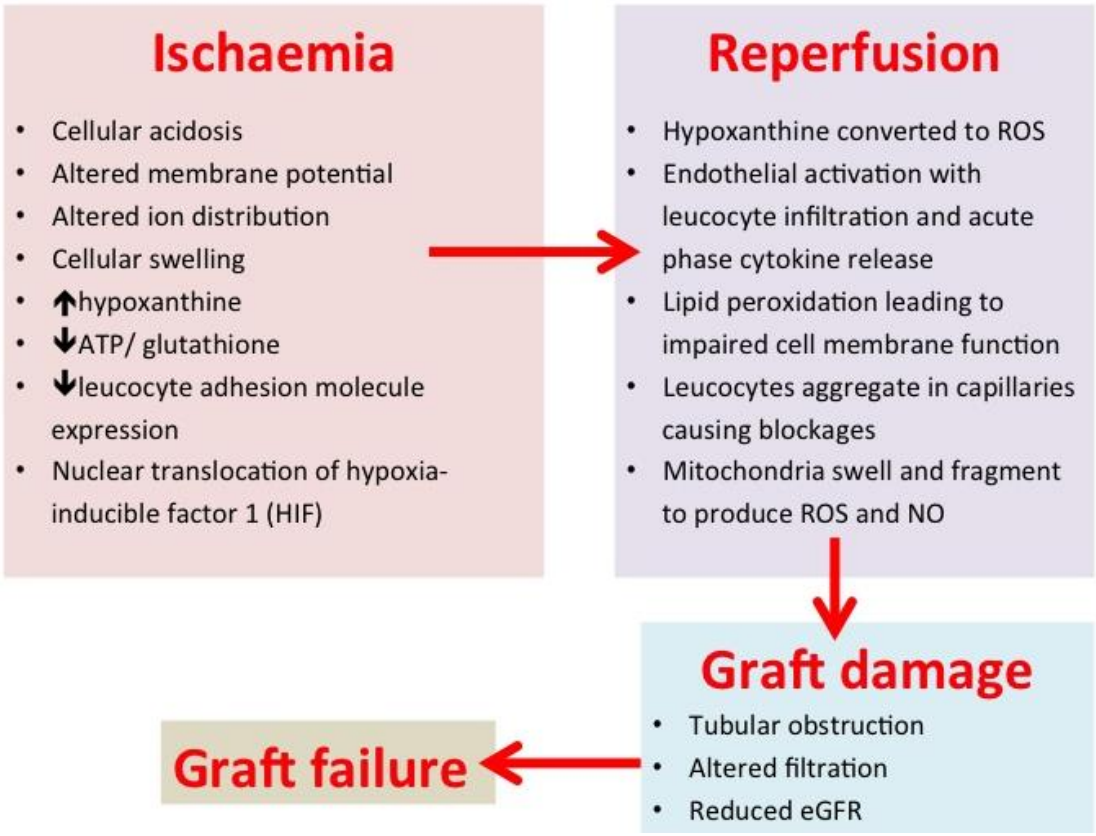


Figure 1- 2 Flow chart of events during ischaemia reperfusion injury (IRI) (adapted from Eltzschig 2004). ROS = reactive oxygen species, NO= nitric oxide

The ability of a graft to protect itself from cellular injury is increasingly proposed as one of the key determinants of graft outcome, and various approaches have been proposed as potential therapeutic interventions. The genotype of a donor or recipient may influence an organ's ability to counter cellular injury and endogenous cytoprotective molecules could have a role in altering the impact of IRI (Courtney and Maxwell 2008). Both of these factors may influence the long-term survival of transplanted kidneys and will be expanded below.

1.3 The macrophage in renal IRI

In the normal kidney, the most prevalent leucocytes are macrophages and dendritic cells and they exhibit a diverse range of roles including phagocytosis, antigen presentation and cytokine production. As a result, they have important functions in innate and acquired immunity, renal injury and inflammation and tissue repair (reviewed by Erwig, Kluth et al 2001).

Blood monocytes are the precursors of tissue macrophages and dendritic cells. A proportion of the circulating monocytes migrate into normal renal tissue where they differentiate into resident monocytes and dendritic cells. Blood monocytes participate in the well-recognised process of rolling along the endothelial wall until they meet damaged tissue, into which they rapidly migrate in response to chemokines and cytokines released by activated endothelial, epithelial and mesenchymal cells (Erwig, Kluth et al. 2001). In response to endogenous inflammatory signals, the monocytes differentiate into macrophages. The resident monocytes and macrophages inhabit the interstitial extracellular compartment, an ideal location to respond to signals from either intravascular substances or surrounding cells (Cao, Wang et al. 2013).

As mentioned in section 1.2, renal IRI is associated with a deleterious influx of polymorphonuclear leucocytes, macrophages and lymphocytes (Kelly, Williams et al. 1996, Lee, Huen et al. 2011). Macrophages are responsible for the sterile inflammation associated with reperfusion either directly from production of pro-inflammatory cytokines such as IL-6, IL-12, IL-18 or indirectly though activation of

NKT cells and T lymphocytes (Li and Okusa 2010). Macrophage infiltration also correlates closely with cell apoptosis (Kluth, Erwig et al. 2004). Early macrophage depletion experiments supported this destructive hypothesis, because pre-treatment of animals with clodronate prior to IRI resulted in a reduction of pro-inflammatory markers and improvements in renal structure and function (Day, Huang et al. 2005, Jo, Sung et al. 2006). However, more recent research offers a more complex picture of macrophages in renal IRI. In an animal model of IRI, Lee et al identified two distinct populations of macrophages. As expected the first cells recruited after IRI were pro-inflammatory and pro-apoptotic and their depletion prior to IRI reduced subsequent kidney injury. But, macrophages that appeared 3-5 days after IRI aided renal tissue repair and their depletion resulted in worse renal injury (Lee, Huen et al. 2011).

Functionally, macrophages are often defined as M1 or "classically activated macrophages" and M2 or "alternatively activated phenotype". M1 macrophages are immune effector cells, which secrete high levels of pro-inflammatory cytokines including TNFα and IL-6. In contrast, the later, M2 phenotype is less well defined but is involved in wound healing and tissue repair. The M2 phenotype is induced in response to IL-4 and IL-13 and results in upregulation of arginase, mannose receptor and MHC II, which go on to stimulate endocytosis, antigen presentation and endothelial cell proliferation (Cao, Wang et al. 2013). Although this model is probably simplistic, it highlights the potential plasticity of macrophages in different inflammatory settings. Li suggests that the different phenotypes derive from two mechanisms; differentiation of separate subsets of circulating monocytes and also

macrophage switching between types in response to cues from the local microenvironment (Li and Okusa 2010).

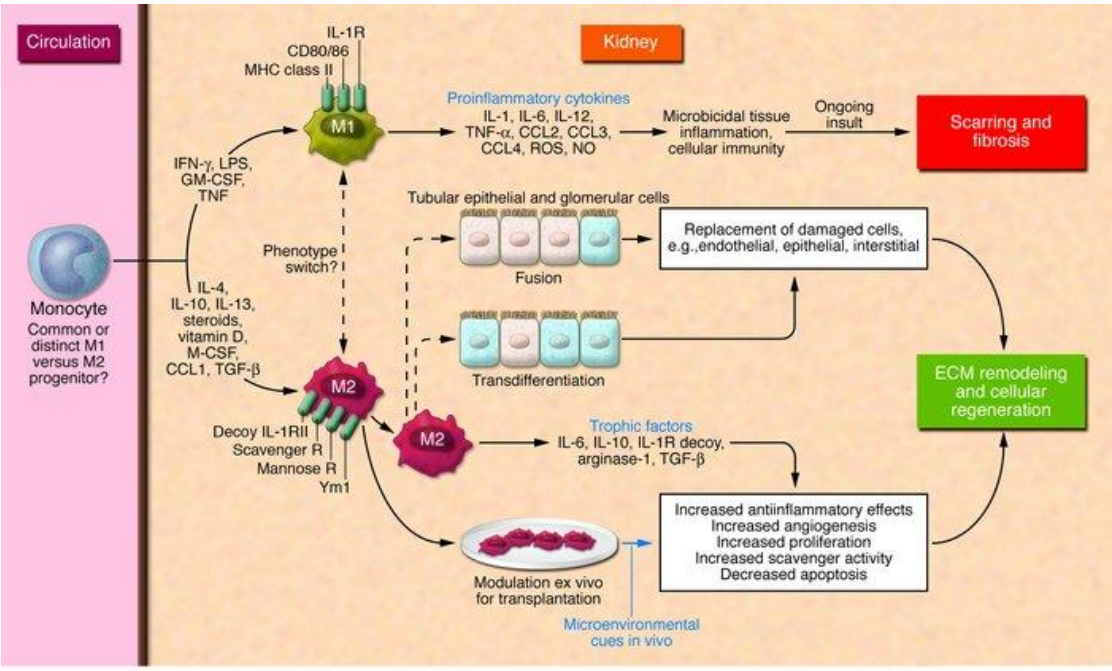


Figure 1- 3 Macrophage differentiation in renal IRI (from Ricardo et al, 2008)

Macrophages are involved in both inflammation and repair in IRI making them a potential therapeutic target, in particular by augmenting their reparative qualities.

1.4 Heme oxygenase-1

As discussed in section 1.2, IRI is characterised by the generation of reactive oxygen species (ROS) and increased production of pro-inflammatory mediators. Given that endogenous antioxidants are depleted when ischaemic organs are reperfused, it has been proposed that treatment with antioxidants might ameliorate IRI. While treatment with synthetic antioxidants has had success (Land, Schneeberger et al. 1994), the utilisation of endogenous antioxidants has also been explored.

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme in the catabolism of harmful pro-oxidative and pro-inflammatory heme molecules, which are released ubiquitously when cells are damaged (Wagener, van Beurden et al. 2003, Ferenbach, Kluth et al. 2010). However, the influence of HO-1 extends beyond reducing cellular heme. In addition to its antioxidant and anti-inflammatory effect, HO-1 also has anti-proliferative, immunomodulatory and anti-apoptotic properties and has a role in regulating vascular tone, thereby protecting the vulnerable microcirculation (Amersi, Buelow et al. 1999, Kinderlerer, Pombo Gregoire et al. 2009, Richards, Wigmore et al. 2010).

It is accepted that HO-1's protective effect is mediated by the products of heme degradation; iron, carbon monoxide and biliverdin/bilirubin, as reviewed by (Courtney and Maxwell 2008) and (Kirkby and Adin 2006).

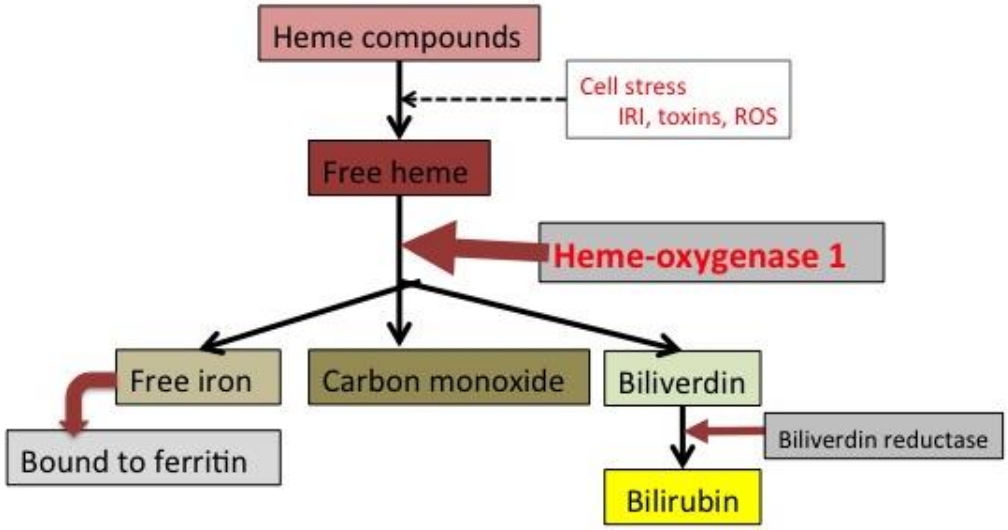


Figure 1- 4 Heme breakdown catabolised by HO-1 (adapted from Ferenbach 2010)

Hill-Kapturczak accurately summarises the situation when she states that the constituents of the HO-1-catalysed reaction are a "Dr Jekyll and Mr Hyde- like phenomenon" (Hill-Kapturczak 2002) because each of the molecules involved possess injurious attributes in addition to their cytoprotective effects (Farombi and Surh 2006).

- **Carbon monoxide** (CO) is well known for its toxic effects but at low levels it behaves as a regulatory molecule in many different processes. CO stimulates the production of cGMP, which modulates various functions such as vascular smooth muscle relaxation and inhibition of platelet aggregation (Brune and Ullrich 1987). CO, in coordination with HO-1, has a role in nitric oxide (NO) regulation and limits NO's free radical formation while promoting its positive vasodilatory effects (Thorup, Jones et al. 1999, Kirkby and Adin 2006).

CO also stimulates the p38 mitogen-activated protein kinase (MAPK) signalling pathway, which is a system involved in responding to stress signals. Its activation results in reduced pro-inflammatory cytokines including TNFα and increased anti-inflammatory cytokines such as IL-10 both *in vitro* and *in vivo* (Brouard, Otterbein et al. 2000). Pro-inflammatory macrophages are also inhibited by this mechanism (Otterbein, Bach et al. 2000) and further immunomodulation can occur via CO mediated T-cell suppression (Pae, Oh et al. 2004).

The cellular mechanisms stated above have successfully translated into pre-clinical experiments. Rats exposed to CO before and after transplant had decreased inflammatory mediators, improved renal blood flow, preserved renal structure and increased survival when compared to control rats (Neto, Nakao et al. 2004). Despite the interest, human studies are scarce. As of July 2014, a trial investigating inhaled CO as a potential treatment for transplant recipients was reported on clinicaltrials.gov as suspended pending protocol changes. Although there are no current human trials investigating water-soluble CO–releasing molecules (CORMs), results from animal studies look promising (Song, Hoeger et al. 2010).

- Paradoxically, even though **bilirubin** is harmful to the developing neonatal brain (Dore, Takahashi et al. 1999), it is also one of the most powerful endogenous antioxidants scavenging peroxyl radicals and inhibiting lipid peroxidation (Stocker, Yamamoto et al. 1987).

It may also have a role in modulating NO production, which can be both beneficial and harmful in IRI. In a rat model of IRI, bilirubin was responsible for inhibition of leukocyte adhesion and rolling after upregulation of HO-1 with hemin (Hayashi, Takamiya et al. 1999). The precursor to bilirubin, **biliverdin**, may also prevent harmful complement deposition (Nakagami, Toyomura et al. 1993).

Bilirubin has also been shown to offer protection in an experimental model. When isolated rat kidneys were perfused with micromolar doses of bilirubin before 20 minutes of warm ischaemia, there were improvements in urine output, eGFR, tubular function and histological appearance compared to the control group (Adin, Croker et al. 2005).

- HO-1 moderates the potentially toxic free **iron** by altering iron mobilization (Ferris, Jaffrey et al. 1999) and co-inducing ferritin, which binds to iron and shields its pro-oxidant and apoptotic effect (Balla, Balla et al. 1992, Kinderlerer, Pombo Gregoire et al. 2009).

HO-1, perhaps in conjunction with CO, also protects against inflammation via down-regulation of adhesion molecule expression (Soares, Seldon et al. 2004) and suppression of monocyte chemoattractant protein 1 (MCP-1), thereby reducing recruitment and binding of leucocytes (Nath, Vercellotti et al. 2001). In addition to its chemoattractant properties, MCP-1 also facilitates the infiltration of monocytes

into the renal interstitium and has pro-inflammatory and pro-coagulant properties (Courtney and Maxwell 2008).

Besides inducing vasorelaxation, HO-1 also acts on vascular smooth muscle to limit its proliferation thereby protecting the microcirculation (Peyton, Reyna et al. 2002).

HO-1 also limits unwanted apoptosis through a variety of mechanisms including increased expression of the anti-apoptotic genes Bcl-2 and Bag-1 (Katori, Buelow et al. 2002) and by upregulation of the cyclin-dependent kinase inhibitor p21 thereby enabling renal cells to resist apoptosis (Inguaggiato, Gonzalez-Michaca et al. 2001).

In another illustration of the importance and adaptation of HO-1, the enzyme is vital for myeloid cell maturation and differentiation (Wegiel, Hedblom et al. 2014) and drives polarization towards the anti-inflammatory, M2 macrophage phenotype (Weis, Weigert et al. 2009, Araujo, Zhang et al. 2012).

As regards transplantation, it is hypothesised that, in addition to HO-1's role in helping the kidney resist ischaemic injury, HO-1 can also modulate acute rejection as seen in a cardiac xenograft model (Soares, Lin et al. 1998). Otterbein hypothesised that the anti-rejection effect is probably due to altered platelet function, vasodilation and reduction in inflammation as suggested in IRI pathology (Otterbein, Bach et al. 2000). Another proposed mechanism is via suppression of the immune response through deletion of autoreactive CD4+ T cells (McDaid, Yamashita et al. 2005).

HO-1 may also have a role in immunomodulation via regulation of HO-1 specific CD8+ regulatory T cells (Andersen, Sorensen et al. 2009).

It is likely that a coordinated response from HO-1 and its reaction components will provide maximal defence against transplant IRI (Kirkby and Adin 2006). This is supported by experimental findings from Nakao et al, who found that dual treatment of rat transplant recipients with biliverdin and CO improved graft function and recipient survival compared with monotherapy (Nakao, Neto et al. 2005).

1.5 Heme oxygenase-1 in renal IRI and transplantation

The HO-1 pathway is important to transplantation because there is evidence that HO-1 upregulation protects the kidney from ischaemia. As the kidney is not normally involved in the breakdown of red cells or haemoglobin, it generally has very low levels of HO-1 activity (Pimstone, Engel et al. 1971). However, HO-1 mRNA, protein and enzyme activity increase as an adaptive response to a variety of noxious stimuli that occur during ischaemic renal injury including: heme released from intracellular organelles, nitric oxide, increased transcription factors NF-kB and MCP-1, growth factors and cytokines (Maines, Mayer et al. 1993, Shimizu, Takahashi et al. 2000, Sikorski, Hock et al. 2004). When induced by any of the mechanisms shown in figure 1- 5, HO-1 is expressed predominantly in distal tubular epithelial cells.

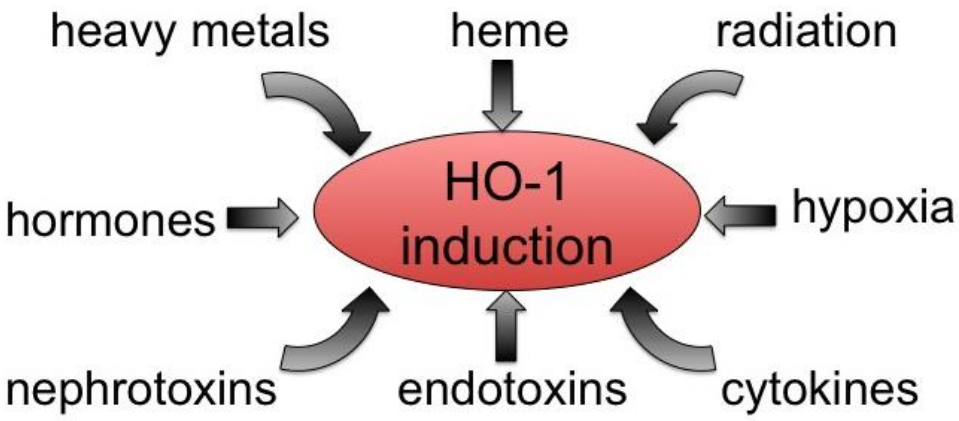


Figure 1- 5 Some of the routes of HO-1 induction

Rat experiments in the 1970s found that renal HO-1 activity increased 30-100 fold after heme injection. Maximum enzyme activity was seen at 6-16 hours with a return to baseline at 48 hours and at its peak, the HO-1 activity of the kidney exceeded that of the spleen (Pimstone, Engel et al. 1971).

In keeping with the renal cytoprotective role of HO-1, graft biopsy specimens with lower levels of HO-1 correlated with increased markers of oxidative stress (Morimoto, Ohta et al. 2001) and humans who are genetically unable to express HO-1 entirely or who only express lesser amounts have a higher incidence of renal disease (Yachie, Niida et al. 1999, Kawashima, Oda et al. 2002, Exner, Minar et al. 2004). The first case report of a patient with HO-1 deficiency describes a child with a variety of pathologies including anaemia and growth failure but also significant microscopic renal disease, demonstrated by tubular dilatation and atrophy, iron deposition in tubules, endothelial cell injury and inflammatory cell infiltrate (Kawashima, Oda et al. 2002).

The development of a HO-1 knock out mouse in 1997 furthered understanding of the enzyme's importance in renal cytoprotection. The embryos were more sensitive to heme damage and the kidneys of HO-1 -/- mice demonstrated iron deposition in renal tubules and a chronic inflammation profile (Poss and Tonegawa 1997). HO-1 -/- mice experienced significantly worse renal injury and 100% mortality when challenged with heme proteins compared to HO-1 +/+ mice (Nath, Haggard et al. 2000). A similarly severe renal injury was seen when HO-1 -/- mice were treated with the nephrotoxic agent, cisplatin (Shiraishi, Curtis et al. 2000).

In the context of renal transplantation, over-expression of HO-1 in a rat model resulted in prolonged graft survival and improved function after extended ischaemia (Wagner, Cadetg et al. 2003).

A study of human renal transplants found significant upregulation of HO-1 in post-reperfusion biopsies compared with pre-reperfusion biopsies and that the level of HO-1 in the latter was closely related to initial graft function (Ollinger, Kogler et al. 2008). Lemos et al found that HO-1 expression was significantly lower in cadaveric kidneys compared to living donors and confirmed Ollinger's findings that the levels of HO-1 expression correlated with renal function in the first week after transplantation (Lemos, Ijzermans et al. 2003). An example of the complex nature of HO-1 induction and activity was highlighted when Nijboer et al found the opposite configuration of HO-1 gene expression and that the cytoprotection offered by HO-1 only appeared to apply to organs from living donors (Nijboer, Schuurs et al. 2004). The impact of the HO-1 gene polymorphisms in renal transplantation will be discussed in a separate section.

As previously described, macrophages are the predominant HO-1 expressing cells (Roach, Moore et al. 2009). In renal IRI models, increased macrophage HO-1 is associated with improved outcome (Gueler, Park et al. 2007) and depletion of HO-1 macrophages results in a greater tubular injury (Ferenbach, Nkejabega et al. 2011). Macrophages that overexpress HO-1 display fewer inflammatory characteristics including reduced TNFα production. This can be replicated by treatment with CO both *in vitro* and *in vivo* (Otterbein, Bach et al. 2000).

There are multiple pathways in transplant IRI that could be beneficially modified by increased HO-1 expression. Thus, it was hypothesised that increasing graft and macrophage HO-1 expression would be a therapeutic approach to reduce the impact of transplant-associated IRI.

1.6 HO-1 induction in renal IRI and transplantation

In general, induction of HO-1 results in structural or functional protection, whilst inhibition has the opposite effect. As expected, given the intrinsic importance of HO-1, the mechanism is not quite this simple because there are other interlinking pathways that are also induced and inhibited in tandem.

The first evidence of the protective effects of HO-1 induction was in a rat rhabdomyolysis model when Nath et al used haemoglobin to induce HO-1 in the renal tissue. They found that increased levels of HO-1 preserved renal function and reduced mortality despite substantial kidney injury (Nath 1992). Since then, a wide variety of mechanisms and compounds have been used in animal renal IRI studies including cobalt, tin, and heat-shock preconditioning to successfully induce HO-1 with protective effects (Redaelli, Wagner et al. 2001, Toda, Takahashi et al. 2002, Matsumoto, Makino et al. 2003, Nath 2006). Induction of CO by methylene chloride had a similar positive effect with improved graft function and reduced chronic rejection (Martins, Reuzel-Selke et al. 2005).

In a model of ischaemic renal injury, administration of cobalt chloride pre- and post-ischaemia increased expression of hypoxia-related genes including HO-1, and resulted in reduced tubular damage, reduced macrophage infiltration and improved function as seen by reduced creatinine levels. HO-1 levels were elevated for up to 48 hours after injury and therefore suggested that HO-1 may have a role in recovery as well as protection (Matsumoto, Makino et al. 2003). Similar results were seen after pre-treatment with tin chloride, which decreased serum creatinine and histological

evidence of injury compared to placebo. Correspondingly, when the HO-1 activity was blocked by tin protoporphyrin and heme concentration increased, this positive effect was abolished (Toda, Takahashi et al. 2002). A review by Akagi et al highlighted the many protective aspects of induced HO-1 in different models of ischaemic acute renal failure (Akagi, Takahashi et al. 2002).

Tullius et al showed that upregulation of HO-1 in rat renal transplant donors by administration of cobalt-protoporphyrin (CoPP) prior to transplant mitigated against acute rejection, prolonged graft survival and improved long-term function in recipients despite long cold ischaemic times. The upregulation of HO-1 was associated with reduced TNFα and increased IFN-γ and bcl-x mRNA and therefore it was hypothesised that the positive effect may be due to reduced macrophage activation (Tullius, Nieminen-Kelha et al. 2002). Another group found that in the subset of DBD grafts, upregulation of donor HO-1 by CoPP also improved graft survival (Kotsch, Francuski et al. 2006).

Recipient survival was improved and renal injury was reduced when gene transfer of a HO-1 expressing adenovirus occurred during the cold storage phase of transplantation offering further evidence of HO-1 protection in transplant IRI and another potential mechanism (Blydt-Hansen, Katori et al. 2003).

However, despite the significant pre-clinical and observational data demonstrating the potential benefit of HO-1 induction, there have been few agents identified that are suitable for clinical use in recipients of renal transplants.

Curcumin, the active component of turmeric, is under investigation because it has been shown to induce HO-1 in hepatocytes and protect against ischaemia (McNally, Harrison et al. 2006). However, experience in a renal transplant setting has been mixed because, although curcumin improved injury markers and the histological appearance of the kidney, this did not translate to renal protection (Hammad, Al-Salam et al. 2012).

Shoskes et al carried out a randomised, controlled trial in 2005 and found that treating the recipient post-transplant with curcumin (as an oral compound with another bioflavonoid quercetin) improved the early function of renal transplant and reduced acute rejection rates (Shoskes, Lapierre et al. 2005). They hypothesised that this protection was due to HO-1 upregulation because curcumin upregulates HO-1 in human renal proximal tubule cells *in vitro* and the active group had increased urinary HO-1 (Hill-Kapturczak, Thamilselvan et al. 2001). However despite these positive findings, there is no evidence that this has been taken further. A search on clinicaltrials.gov in July 2014 for "curcumin AND transplant" lists only one trial, which is for investigating a curcumin-containing preservation solution but this record has not been active for two years.

Statins also upregulate HO-1 and initially provided an exciting therapeutic option as they are generally safe and well tolerated. However their potential has not been realised as the multicentre ALERT trial showed no positive impact on graft function as measured by eGFR and graft loss over five years (Fellstrom, Holdaas et al. 2004).

In 2001, Salahudeen found that pre-treating human renal proximal tubular epithelial cells with hemin (a form of heme) increased HO-1 mRNA and protein expression and this pre-treatment prevented cold-storage induced injury of the type experienced in deceased donor transplantation (Salahudeen, Jenkins et al. 2001). Yoneya et al used another form of heme, hemolysate, to successfully upregulate HO-1 and found it ameliorated ischaemic acute injury in rats (Yoneya, Nagashima et al. 2002).

In 2008, Holzen used hemin as a pre-treatment agent in donor rats prior to transplant and this resulted in improved recipient graft function when compared to recipients whose donors had not received hemin. There was histological evidence of HO-1 upregulation and a significant improvement in microcirculation perfusion (Holzen, August et al. 2008). In 2011, Li et al also used hemin to upregulate HO-1 in rat transplant recipients with an improvement in renal function and reduction in rejection (Li, Wang et al. 2011).

Our group also found that a form of hemin licensed for human use, heme-arginate (HA), was an effective inducer of HO-1 in murine macrophages and renal tissue and preserved renal function following IRI (Ferenbach, Nkejabega et al. 2011). These experiments showed that aged mice developed more severe renal impairment following IRI than matched younger animals. The aged mice had less HO-1 induction but this could be reversed by treatment with HA, which appeared to protect against IRI. This protective effect was lost when macrophages were depleted. Thus HA's beneficial effects appeared to be mediated via monocyte/macrophages in renal IRI.

The first healthy volunteer trial showed that administration of 3mg kg^{-1} hemin increased the plasma level of HO-1 protein five times and HO-1 activity 15-fold 24 hours after injection (Bharucha, Kulkarni et al. 2010). A follow-on, randomised, placebo-controlled trial confirmed that HA infusion safely upregulated HO-1 in the peripheral blood mononuclear cells (PBMC) of healthy subjects (Doberer, Haschemi et al. 2010).

The review of HO-1 by Nath in 2006 explored the risks of HO-1 induction and, as previously highlighted, acknowledged the potentially harmful nature of the products of heme degradation. It has been suggested that while low-level HO-1 induction is beneficial, it may be detrimental in excess (Suttner and Dennery 1999). Therefore it is important that the optimal level of HO-1 expression is identified to ensure that any alteration in upregulation rates result in protection rather than harm. Understanding the underlying molecular mechanisms controlling HO-1 expression is therefore essential.

HO-1 gene regulation in renal injury is complex with a variety of HO-1 inducers using different pathways in different cell types. There are multiple binding sites on the HO-1 gene for different transcription factors including; Nrf2, nuclear factor KB (NF-κB), specialty protein 1 (Sp1), ATF-2, Elk, Jun, activator protein 1 (AP-1), AP-2, HIF-1, upstream stimulatory factor (USF), TGF-B1 and IL-6 response elements suggesting that any or all of these may play a role in modulating HO-1 induction (reviewed by (Sikorski, Hock et al. 2004, Traylor, Hock et al. 2007, Hsu, Chu et al. 2008, Deshane, Kim et al. 2010).

In the mouse, the protein Nrf2 is vital for regulation of the HO-1 gene and is responsible for HO-1 induction in response to a range of stimuli including heme and is likely to have an important role in humans (Akagi, Takahashi et al. 2002). HO-1 transcription is tightly controlled and Bach1 is a transcriptional repressor whose expression is inversely regulated with HO-1. Further analysis has shown that Bach1 provides a compensatory mechanism to prevent overexpression of HO-1 in a variety of cell types *in vitro* including skin fibroblasts (Raval, Zhong et al. 2012) and monocytes (Paine, Eiz-Vesper et al. 2010). It has been proposed that, at low heme concentrations, HO-1 is repressed by Bach1, but at higher concentrations, the heme binding sites of Bach1 are inactivated allowing access to a variety of transcription factors such as Nrf2 with downstream gene activation (Ogawa, Sun et al. 2001).

Closer investigation of the human HO-1 promoter gene identified two regions that are responsible for HO-1 transcription in response to heme (Hill-Kapturczak, Sikorski et al. 2003). One of these was identified as a binding site for Jun proteins with JunB acting as an activator of HO-1 promoter activity and JunD as an inhibitor (Hock, Liby et al. 2007). The same group also identified Sp1 as regulating functional activity of the HO-1 gene in renal cells in response to heme (Deshane, Kim et al. 2010).

Gene regulation in human monocytes/macrophages has also been investigated and it is likely that Nrf2 is a key player in regulating expression of many oxidative stress-inducible genes including HO-1 (Ishii, Itoh et al. 2000, Boyle, Johns et al. 2011, Rushworth, Shah et al. 2011).

The varied means of HO-1 induction from different inducers makes the process complicated and it is clear that this will continue to be explored to try to elicit areas of therapeutic intervention.

The overarching theme for HO-1 induction is that it requires a small noxious stimulus, which then confers protection against a more substantial IRI insult. This is the essence of the pre-conditioning phenomenon, which is explored further in section 4.2.1 of the discussion.

1.7 Heme oxygenase-1 phenotype

HO-1 inducibility is modulated by a dinucleotide guanosine thymine (GT) repeat polymorphism within the proximal promoter region of the HO-1 gene. Although the reported number of repeats varies from 12- 40, most human populations have a bimodal distribution with alleles being either 23 (short or S) or 30 (long or L) repeats (Exner, Minar et al. 2004). There is evidence that long repeats are associated with less HO-1 gene expression *in vitro* in comparison to short repeats (Chen, Lin et al. 2002) and this is associated with susceptibility to a variety of diseases including coronary artery disease, aortic aneurysm and asthma, reviewed by (Doberer, Haschemi et al. 2010).

Initial work suggested that this phenotypic difference translated to improvements in transplant outcomes because recipients who received organs from S-allele donors had lower serum creatinine at one year follow-up (Exner, Bohmig et al. 2004) and improved graft survival at five year review (Baan, Peeters et al. 2004). However, these genetic association studies have proved difficult to replicate and the phenotype incidence varies across populations. A larger study found no evidence that donors or recipient genotype had a significant impact on graft or recipient survival in Northern Irish populations (Courtney, McNamee et al. 2007) and this has been corroborated across different populations including Greece (Katana, Skoura et al. 2010) and Finland (Turpeinen, Kyllonen et al. 2007). In fact, the established *in vitro* results were not reproduced in either the larger study by Zhang et al where longer GT_n repeats did not inhibit HO-1 promoter activity (Zhang, Ohta et al. 2006) or the first healthy volunteer study.

Doberer treated healthy volunteers with HA and upregulated HO-1 in PBMCs but the GT_n length polymorphism had no effect on the extent of HO-1 induction. In fact, they found the opposite, with HO-1 mRNA higher in the L/L group. They proposed that this finding was due to either differences in *in vivo* or variation in HO-1 transcription in response to heme. Doberer also suggested that different cell types may respond to HA differently and further PBMC subpopulation analysis may be required (Doberer, Haschemi et al. 2010). Courtney et al hypothesised that there are other protective gene variants or molecules in renal transplantation, which may compensate for a genetic predisposition towards lower HO-1 production. This ensures that even if the HO-1 genotype could alter the cellular response to injury, it may be masked clinically (Courtney and Maxwell 2008).

1.8 Hemin and heme arginate

As discussed previously, although heme is directly toxic to adjacent cells, at non-toxic concentrations heme and its derivatives deliver potent preconditioning stimuli that protect *in vitro* cells from subsequent exposure to toxic conditions (Balla, Jacob et al. 1993, Regan, Guo et al. 2000, Li, Zhu et al. 2009). The protective action has a lag of several hours and this may explain why the HOT study is the first translational study to use hemin or heme arginate (HA) as a pre-conditioning agent (Balla, Balla et al. 1992).

Hemin is the ferric form of heme with a chloride ligand whereas HA (or hematin in the United States) is ferric heme with a hydroxide ligand and it is this form that has been licensed for use in acute porphyrias for over 30 years with few side-effects (Lu, Chen-Roetling et al. 2014). In the UK, it is supplied by Orphan Europe under the proprietary name of Normosang.

The chemical formula of HA is;

$C_{34}H_{18}FeN_4O_4$ or (chloro {7, 12-diethenyl-3, 8, 13, 17-tetramethyl-21H, 23H-porphine-2, 18-dipropanoato (4-)-N^{21}, N^{22}, N^{23}, N^{24}} ferrate (2-) dihydrogen) and the structural formula is shown in figure 1- 6.

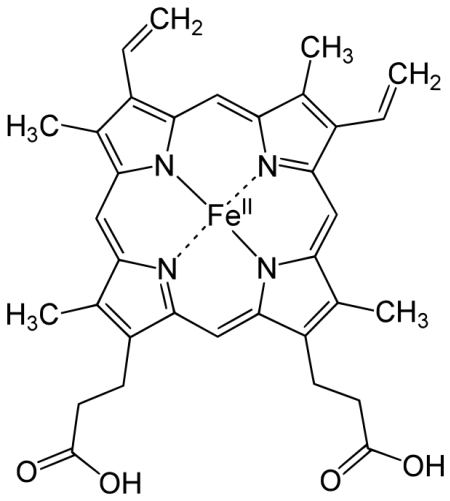


Figure 1- 6 Heme arginate molecule

Most of the reported adverse events for hemin are attributed to the reaction products of ferric heme and this can be reduced by liganding heme to arginine as in HA (Tenhunen, Tokola et al. 1987). The most common reported side effect of HA is infusion site irritation and swelling due to the hypertonic nature of the solution. To counteract this, it is advised that infusions are given into large veins or via central access. There have been three recorded cases of overdose; in all three cases, the patients received 10 times the recommended amount and all suffered liver failure. One required a transplant (http://www.medicines.org.uk/EMC/medicine/20795/SPC/Normosang/).

After injection, HA rapidly binds to high affinity heme binding sites mainly on albumin and is excluded from most organs (Linden, Tokola et al. 1987). It is therefore thought that its primary action is likely to be on vasculature (Lindenblatt, Bordel et al. 2004, Belcher, Mahaseth et al. 2006, Holzen, August et al. 2008).

In the treatment of porphyria, hematin and HA act by inhibiting of δ-aminolevulinic acid synthase rather than the previously discussed mechanism of HO-1 induction (Anderson and Chapman 2005).

Hemin has been of interest for decades; systemic pre-treatment with hemin has been shown to be protective in many acute injury models, including kidney, brain, heart, gut, and pancreatitis (reviewed in Lu et al 2014) but few have translated to clinical studies.

Correa-Costa found that when mouse kidneys were treated with hemin, 346 genes were upregulated, including those involved in transcription regulation and DNA repair. When the microarray was repeated after renal IRI following hemin pre-treatment, the gene expression profile altered with increased expression of genes responsible for vascular smooth muscle contraction and arachidonic acid metabolism and reductions in apoptotic genes (Correa-Costa, Azevedo et al. 2012). It was hypothesised that this change in transcriptome may be mediated by HO-1 (Lu, Chen-Roetling et al. 2014). Alternatively, hemin may have a direct regulatory effect on transcription factors and signal transducers including Bach 1 and MAPK pathways (Mense and Zhang 2006). Further research is required to elicit the mechanisms through which heme gives protection.

1.9 The HOT Study

In conclusion, HO-1 induction limits cell injury *in vitro*, reduces kidney injury and protects against IRI in animal transplantation models. HA can safely upregulate HO-1 in humans and elevated levels of HO-1 improve outcome after renal transplant.

Against this background, the team at University of Edinburgh developed a clinical trial to translate these bench findings to bedside.

The aims of the HOT (Heme Oxygenase-1 in renal Transplantation) study were:

- to determine if pre-treatment of recipients of deceased donor renal transplants with HA was feasible and safe.
- to investigate if HO-1 could be upregulated in the recipient macrophages and renal graft tissue by HA.
- to look at the effect of HA on urinary biomarkers, graft histology and graft function.

Chapter 2.

Materials and Methods

2.1 HOT study logistics

The HOT (Heme Oxygenase-1 in renal Transplantation) Study was a single-blinded, randomised, placebo-controlled trial. It was registered on the European Clinical Trials database (EudraCT no: 2011-004311-23) and at ClinicalTrials.gov (no: NCT01430156).

The aim of the study was to investigate heme oxygenase-1 (HO-1) upregulation in the macrophages and renal tissue of renal transplant recipients in response to heme arginate (HA) infusion. The study took place in the transplant unit in Royal Infirmary of Edinburgh (RIE), recruiting its first participant in January 2012 and continuing until 40 participants had been recruited in May 2013.

2.1.1 *Trial aims*

In line with CONSORT guidelines, the trial team's first task was to define the HOT Study objectives and end-points. It was from this starting point that the HOT study developed.

2.1.1.1 Primary objective

Does pre-treatment of recipients with HA prior to renal transplantation increase HO-1 protein expression in the recipient monocytes/macrophages vs. placebo at 24 hours?

2.1.1.2 Secondary objectives

Does pre-treatment of recipients with HA prior to renal transplantation increase HO-1 mRNA in the recipient monocytes/macrophages compared to placebo at 24 hours?

How does monocyte HO-1 mRNA and protein expression change over five days after HA/ placebo infusion?

Does pre-treatment of recipients with HA prior to renal transplantation and post-operatively increase HO-1 mRNA and protein expression in the transplanted kidney vs. placebo at day 5?

Does HA treatment reduce histological acute tubular injury in renal allograft vs. placebo? To include assessment of acute tubular necrosis (ATN) scores, infiltrating leucocyte populations (macrophages, neutrophils, T and B cells), endothelial cells, presence of platelet microthrombi and tubular proliferation on the biopsy specimens.

Does HA improve outcome after transplantation by reduced delayed graft function rates in HA group compared to placebo?

Can we predict the amount of injury a transplanted kidney has sustained by measuring biological markers in the urine?

2.1.2 *Trial funding*

NHS Blood and Transplant funded the HOT study and the money was secured before the author started at the University. As Chief Investigator (CI), the author was responsible for developing the trial concept and logistics, writing the protocol, naming the trial, applying for approvals and running the study but not a grant holder.

2.1.3 *Sample size and recruitment calculation*

CONSORT state that a sample size calculation should be performed for all clinical studies (http://www.consort-statement.org/checklists/view/32-consort/66-title). As there was limited data in this field, the calculation was based on a small (n=3) *in vitro* experiment of observed HO-1 upregulation in human macrophages following HA treatment. It was presumed that the pre-treatment value would be similar to the baseline measurement and the 20μM HA treatment was equivalent to 3mg kg^{-1} IV HA treatment. It was also assumed that the responses were paired. The trial statistician used the mean and standard deviation (sd) to calculate the numbers required.

Using a two-sided two-sample test with a 5% level of significance and 80% power, assuming a mean baseline to 24-hour change of 7.47 and a common sd of 3.87, the minimum detectable difference in means would be 3.8 corresponding to a sample size of 17 per group. The final sample size of 20 per group allowed for dropouts.

Audit data from the RIE transplant unit showed that 75 cadaveric renal transplants had been performed between April 2009 and May 2010. Using an anticipated recruitment of 60% of all potential participants, it was expected to take approximately 12 months to complete.

2.1.4 *Trial approval*

The study was planned in line with the principles of the International Conference on Harmonisation Tripartite Guideline for Good Clinical Practice (ICH GCP). All approval applications were submitted through the IRAS online application system.

After meeting with the Scotland Regional Ethics Committee, a favourable ethical opinion was obtained. The study received Clinical Trial Authorisation (CTA) from the Medicine and Healthcare Products Regulatory Agency (MHRA) for a phase IIB clinical trial. The HOT study fulfilled IIB criteria because it planned to investigate the efficacy and safety of HA and was not concerned with dosing requirements (IIA criteria). The HOT study protocol v6.3 (appendix A), which provided the instructions and framework for running the trial, complied with the Medicines for Human Use (Clinical Trials) Regulations 2004.

In order to gain approval from NHS Lothian R&D, the trial required agreement from all parties concerned and required negotiations with the research contacts in NHS Lothian Pathology Department, NHS Lothian Radiology Department, NHS Lothian Pharmacy and support services in the Welcome Trust Clinical Research Facility (WTCRF).

In February 2013, the Data Management Committee (DMC) recommended that participant follow-up be extended and it was decided that this change fulfilled substantial amendment criteria as defined by the MHRA. The amendment process was completed successfully and the protocol, patient information sheets, GP letters and consent forms were updated accordingly.

2.1.5 *Trial insurance and indemnity*

The HOT study was sponsored by ACCORD (Academic and Clinical Central Office for Research and Development: a joint company from University of Edinburgh and NHS Lothian; approval number 2011/R/TR/03 and protocol number HOTstudy_Thomas11). The sponsors were responsible for insurance and indemnity to cover their liability and the liability of the CI and other staff.

The University insured the CI and the trial team for negligent harm caused by poor protocol design and NHS Indemnity covered the RIE.

Orphan Europe accepted limited liability related to the manufacturing and original packaging of the study drug and to the losses, damages, claims or liabilities incurred by study participants based on known or unknown adverse events, which arose out of the manufacturing and original packaging of the study drug.

2.1.6 *Trial management*

In signing the Investigator's Declaration, the author as CI became responsible for the overall conduct of the HOT study and thereby guaranteed compliance with the protocol. A delegation log ensured responsibilities could be delegated to another member of staff in the event of CI not being available although this was not required. All study staff completed appropriate Good Clinical Practice (GCP) training.

The Trial Steering Committee (TSC) was established to supervise progress and to review the trial using relevant information from other sources including the DMC. It also ensured that the trial was conducted within the GCP guidelines. The TSC included the CI, fund holders, trial statistician, sponsor representative and an independent Chairperson. The TSC approved the original and all changes to the trial protocol.

The DMC comprised three independent members with links to clinical transplantation. The author provided monthly reports to them and organised the meetings. The role of the DMC was to look at the data from an ethical standpoint with the safety, rights and well being of the trial participants being paramount. They reviewed the safety profile of the study with particular interest in adverse events. To this end, the DMC requested two interim analyses of trial data and after review, were satisfied to recommended continuation of the trial.

2.1.7 *Trial documentation*

The Investigator Site File (ISF) contained all the information about the trial and was written and maintained by the author (contents list in appendix B). Once the trial started recruiting, any protocol deviations were recorded as file notes. The author wrote the data collection forms (DCFs) in collaboration with the trial statistician (appendix C). All DCFs, reports and other records were designed to maintain participant confidentiality and were kept in a secure storage area. Written agreement was required for the disclosure of any confidential information to other parties. All documentation will be stored for five years in accordance with GCP.

The HOT Study database was written by the author and hosted online by the WTCRF (https://www.wtcrf.ed.ac.uk/StudiesV6/Login.aspx?dbid=hot). This method of data storage ensured safe and auditable data management. The digital forms contained the same fields as the original paper DCFs and the response options were limited in order to minimise error during subsequent data entry. Once finished, the database was checked by the trial statistician and locked so that only WTCRF IT staff could access the form templates. Once the trial started, the author entered the data from the DCFs into the database and this underwent quality control at regular intervals by ACCORD staff. The author could alter the data until all results were inputted when the database was locked again for statistical analysis. This prevented anyone from changing results after analysis to ensure transparency.

When it was time for the statistical analysis, independent statisticians used the data dictionary compiled by the author to ensure only the relevant data was exported.

2.1.8 *Trial audit and monitoring*

A Clinical Trials Monitor from ACCORD performed the initial 'green light' assessment at the start of the study and monitored 11 times during the recruitment phase to confirm adherence to GCP. There were also regular assessments of the accuracy of the data collection by an independent reviewer using study records and case notes as source documents.

The University of Edinburgh laboratories were also inspected and audit procedures were put in place. These included calibration of equipment and assessment of external apparatus.

In March 2013, the MHRA carried out a routine inspection of ACCORD and the HOT study was chosen for closer examination. This involved an in-depth review of the ISF and the related documents, DCFs and their source documentation and monitoring paperwork. The author as CI was also interviewed about GCP knowledge and trial behaviour. The inspection found there were no serious concerns about the HOT Study and the three minor recommendations were adopted. No follow-up assessment was required.

2.1.9 *Investigational medicinal product (IMP) arrangements*

Heme arginate (trade name Normosang) was purchased in bulk from the manufacturers Orphan Europe. The drugs were over-labelled with HOT study labels identifying them as trial supplies in accordance with the Medicines for Human Use (Clinical Trials) Regulations 2004 (examples of labels in appendix 8 of protocol v6.3 – appendix A.) HA is licensed for the treatment of acute attacks of hepatic porphyria (acute intermittent porphyria, porphyria variegata, hereditary coproporphyria) and therefore, HA was used outwith the licensed indications.

The HA dose of 3mg kg $^{-1}$ was decided upon as this is the maximal approved dose for treatment of acute porphyria. It is also the dose necessary for transcriptional induction of HO-1 in PBMC (Balla, Jacob et al. 2000). The maximum dose was capped at 250 mg, which is equivalent to one vial and the dosing table is included (appendix 9 of protocol v6.3 – appendix A). Taking advice from the Normosang SmPC, the maximum concentration of HA administered was limited at 2.5mg ml^{-1} in order to minimise the risk of phlebitis and pain at the site of infusion. HA was diluted in 0.9% NaCl and the total volume of infusion was standardised at 100mls for ease and to maintain blinding. The placebo was 0.9% NaCl.

2.1.10 *Personnel coordination*

The HOT study required coordination and cooperation from many different individuals and departments and it is to their credit that the trial ran so successfully. Before the trial started, the study was presented at the unit meeting to inform the

relevant individuals and answer questions. Regular e-mails from the author ensured that the HOT study remained in people's consciousness.

The Transplant Recipient Coordinators, who are experienced professionals responsible for the coordination of the transplant, were responsible for contacting the author when a potential deceased donor renal recipient was on their way to hospital. Once in hospital, the author would liaise with ward nursing staff and the dialysis unit to meet the potential participant. If the patient consented, the theatre team, anaesthetist and surgeon were contacted to make them aware of the recruitment and any potential impact on their work. Biopsies required pathology input and day 5 biopsy requests were made to a named Consultant in the pathology department. The author informed the ward nursing staff and phlebotomy staff about trial participants aided by the transplant research nurse.

2.1.11 *Day to day trial management*

In addition to the actions in 2.1.10, there were regular essential activities once the study started. These included recruiting patients and answering their questions and those of their family, taking blood, administering infusions and undertaking clinical assessment. If participants agreed to biopsies, these were organised and the specimens taken to pathology. Paperwork had to be completed for safety and compliance. In the event of a safety concern, the patient was assessed and reported as appropriate.

In addition to this, there were regular stock checks of the IMP, paperwork, and clinical and laboratory supplies. In the lab, samples were frozen for later batch analysis and the fridges and freezers were temperature checked daily.

Occasionally, there were unexpected events, which required urgent action such as temperature excursions on the ward drug stock fridge.

Every month, a DMC report was prepared. This reported on recruitment info and rates of adverse events.

There were annual reports for the funders, sponsors, MHRA and the Ethics committee. Follow-up data was also collected and collated.

2.1.12 *Randomisation*

The randomisation was done by random, random block, with stratification by donor type. This ensured that there were equal numbers of organs donated after brain death (DBD) and organs donated after circulatory death (DCD) in each group thereby taking into account the most significant risk factor for IRI. The trial statistician generated the random allocation sequence and an independent party produced sequentially numbered, sealed, opaque envelopes, which were only opened by the CI after consent had been given. The date and time of the randomisation was witnessed and recorded. Once opened, the envelopes were stored in a remote secure location.

2.1.13 *Trial safety*

On commencement in the trial, all participants were seen daily during their admission by a member of the trial team and questioned about potential adverse events (AEs). Particular attention was given to the known side effects of HA, listed in the SmPC (appendix 1 of protocol v6.3- appendix A).

The HOT study used the following GCP definitions.

- An **adverse event** (AE) is any untoward medical occurrence in a clinical trial participant, which does not necessarily have a causal relationship with the IMP, (HA in the HOT study).
- An **adverse reaction** (AR) is any untoward or unintended response to IMP, which is related to any dose administered to that participant.
- An **unexpected adverse reaction** is an adverse reaction that is not consistent with the SmPC for IMP.
- A **serious adverse event** (SAE), **serious adverse reaction** (SAR) or **suspected unexpected serious adverse reaction** (SUSAR) is any AE, AR or UAR that at any dose:
    - results in death;
    - is life threatening;
    - requires hospitalisation or prolongation of existing hospitalisation*;
    - results in persistent or significant disability or incapacity;
    - is a congenital anomaly or birth defect.

* Given that all patients in the HOT study were hospitalised and left the trial at discharge, only prolongation of stay was applicable to the HOT study.

Many of the participants were in poor health pre-operatively and certain post-operative events were expected so the decision was made to limit the AEs recorded. These exemptions are recorded in protocol v6.3 (appendix A). The author was responsible for the detection and documentation of AEs and participants were instructed to contact medical staff with any new symptoms (AE reporting log form, appendix D).

If any AE met the regulatory criteria of SAEs, it was reported in detail in that day's DCF and on a separate SAE form (appendix E). The author assessed for seriousness, expectedness and causality as defined in the protocol. Any AEs/SAEs judged as having a reasonable suspected causal relationship to HA were considered as related and therefore classified as adverse reactions or serious adverse reactions (ARs/SARs).

In line with GCP, SAEs were reported to the sponsors within 24 hours. After recording an AE or recording and reporting an SAE, the participant was followed up until the end of the trial. SAEs still present in participants on the day of discharge from the trial were monitored until resolution of the event or until no longer medically indicated and a follow-up SAE report was completed. The ACCORD Research Governance & QA Office reported SAEs to the co-sponsors (Edinburgh University and NHS Lothian), the competent regulatory authority and relevant ethics committee.

In the event of a SUSAR occurring when the CI was unavailable, a pharmacist could authorise an emergency unblinding and for this reason, the pharmacy department held copies of the patient's randomisation status in sealed envelopes.

All staff complied with the requirements of the Data Protection Act 1998 with regard to the collection, storage, processing and disclosure of personal information. Access to collated participant data was restricted to those clinicians treating the participants. Computers were password protected and did not contain patient identifiable data.

2.1.14 *End of Study*

The end of the active component of the study was defined as post-operative day 7 for the 40th participant. However, the follow-up blood results at day 30 and day 90 were recorded in line with the DMC request.

The end of the study was reported to the REC and MHRA within the 90-day limit and the summary reports were provided within the 1 year time limit.

2.2 Clinical components of HOT Study

2.2.1 *Clinical interactions*

In December 2011, all patients on the East of Scotland kidney transplant waiting list received a letter about the trial (Patent Information Sheet- appendix 5 in protocol v6.3; appendix A). Another mailing was sent to all new additions at trial midpoint. The author was available during office hours to answer patient questions and some patients made contact to discuss the trial and its impact. Patients had the option to opt out of the trial in advance. The author met all of the remaining patients once they arrived in RIE for their transplant to assess for eligibility.

All RIE transplant patients are over 18 and there was no upper age limit to the HOT Study. A patient was ineligible for inclusion in the study if:

1. they were unable to receive the standard immunosuppressive regime
2. they were unable to give informed consent
3. they had a known hypersensitivity reaction to HA
4. they were receiving more than one organ
5. it was their 3rd or subsequent kidney transplant
6. they were fully anticoagulated pre-operatively
7. they were on combined anti-platelet agents

Patients who fulfilled criteria 6&7 were exempt to reduce the risk of bleeding complications from the day 5 renal biopsy.

All potential participants who met inclusion criteria had consort data recorded anonymously including gender, age, type of transplant, and reason for ineligibility or non-recruitment. This was recorded on the initial contact form (DCFs; appendix C).

Signed informed consent was obtained and the participant received a copy (appendix 4 in protocol v6.3; appendix A). A letter was also sent to the participant's GP to inform them of the participation (appendix 6 in protocol v6.3; appendix A). Patients could withdraw from the study at any point without affecting their treatment.

Once the patient had consented, they were assigned a participant number and randomised by opening the corresponding envelope. In order to eliminate bias, consent preceded randomisation. The appropriate drug was then prepared.

There were two arms in the study:

- **Arm A**

This group received an infusion of 3mg kg^{-1} HA diluted in 100ml 0.9% saline over 30 minutes into a large peripheral or central vein prior to transplant surgery and again, on day 2. Both infusions were followed by another 100mls of saline to flush the cannula and vein.

- **Arm B (control group)**

This group received an infusion of 100ml 0.9% saline over 30 minutes into a large peripheral or central vein prior to transplant surgery and again, on day 2. Both infusions were followed by another 100mls of saline to flush the cannula and vein.

HA stock was held in RIE pharmacy and in a secure fridge in the transplant unit for administration out of hours. HA is a dark green solution and therefore, to ensure blindness, all infusions were administered through opaque bags and giving sets. Only the author, who was responsible for administering the trial drug, was aware of the nature of the infusion. Once randomised, the infusion was prepared and given immediately.

Blood samples were taken to measure the HO-1 expression in circulating macrophages/monocytes immediately prior to transplant (baseline), 24 hours later (day 1), prior to the second dose of HA/placebo (day 2) and day 3 and day 5 post-transplant. This mapped HO-1 expression over time. The window for the first post-transplant blood sample was 20-28 hours post infusion to avoid patient disruption overnight and allow for staff work planning. Once the blood had been taken, the samples were processed in the Queen's Medical Research Institute (QMRI).

Two renal biopsies were performed: prior to implantation on day 0 (baseline) and day 5 (the recommended time for diagnostic renal biopsy). The pathologist examined the specimens for renal injury and HO-1 induction was determined using conventional laboratory investigations.

Daily, early morning urine samples were collected to detect urinary biomarkers.

All participants received standard post-operative care and standard immunosuppressive triple therapy.

2.2.2 *Clinical data collection*

All participants had a HOT Study checklist completed to ensure that the appropriate trial procedure was followed and fulfilled (DCFs- appendix C).
Background information was obtained from the patient and their medical notes. It included (but was not limited to) participant weight, indication for transplant, number of previous transplants, current medication, smoking status and alcohol history**.** The need and timing of pre-operative dialysis was also recorded.

Donor data collected included type of donor (DCD vs. DBD), medication, cause of death and ischaemic times. This information was available from the transplant coordinator.

Information was also gathered from the operation note and the anaesthetic chart. This included cold and warm ischaemic time, quality of reperfusion, ASA grade, the duration of surgery with the estimated blood loss, minimum blood pressure and the need for transfusion. Each infusion had a DCF, which recorded the timings and completeness of the infusion.

The participants were reviewed daily to monitor recovery and for evidence of post-operative complications. This data and relevant blood results were recorded on DCFs. Renal recovery was assessed by the daily serum creatinine and the need for dialysis.

In the HOT study, delayed graft function (DGF) was pre-defined as an increased or stable serum creatinine, or a decrease of less than 10% per day in 3 consecutive days in the first week after transplantation. Participants were followed up for seven days post-transplant or until discharge from hospital, whichever was sooner. Participant journey is shown in figure 2- 1.

2.2.3 *Clinical follow-up*

The DMC recommended that follow-up blood results for all participants be recorded including day 30 and day 90 creatinine, eGFR, haemoglobin (or as close to these dates as possible.) Readmissions within 30 days of transplant and dialysis status were also recorded. This information did not require additional patient contact and all participants consented to provision of additional information collection.

Figure 2- 1 HOT Study participant time plan

December 2011	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 7**
Patients contacted by letter to inform them of trial Questions answered by team	Patient contacted about suitable transplant and attends RIE Patient **consents** to trial **1st blood test** **Urine sample** **1st dose HA/placebo** **Pre-implantation renal biopsy** Participant receives **transplant**	Routine observation Routine blood tests **2nd blood test** (24hrs post infusion) **Urine sample**	Routine observation Routine blood tests **3rd blood test** **Urine sample** **2nd dose HA/placebo**	Routine observation Routine blood tests **4th blood test** **Urine sample**	Routine observation Routine blood tests	Routine observation Routine blood tests **5th blood test** **Urine sample** **Renal biopsy**	Discharge from study

2.3 HOT Study sample analysis

All the processes below were developed as Standard Operating Procedures (SOPs) and approved by QMRI management and ACCORD staff in line with Good Laboratory Practice (GLP) guidelines for CTIMPs. All documentation was available in the Investigator Site File (ISF) for the HOT Study.

Samples were stored anonymously, only identifiable by a unique, sequential trial number. Participant samples were stored in an individually labelled box in the -80^0C freezer, for which the temperature was checked and logged. All histological slides were labelled with the trial number and stored at constant room temperature in a secure cupboard. All reagents used in the HOT study analysis were stored in a fridge at 2-4°C, for which the temperature was checked and logged.

As the author also performed the laboratory analysis, a further blinding process was required to eliminate any potential bias. To this end, an independent lab member blinded the samples prior to analysis. Once the samples had been analysed, the laboratory results were entered into the database under the blinded number. At the end of the trial, the independent lab member sent the statistician the blinding code to allow subsequent unblinding.

2.3.1 *Peripheral blood mononuclear cells (PBMC) analysis*

PBMC were extracted from whole blood using the Dextran and Percoll gradient method (de Almeida, Silva et al. 2000).

2.3.1.1 Method for PBMC extraction from whole blood

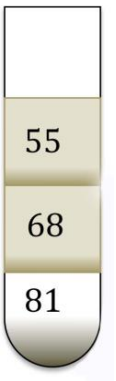
40ml of whole blood was collected by participant venepuncture and transferred to a 50ml Falcon tube containing 4ml of chelating agent (3.8% sodium citrate). After mixing, the anti-coagulated blood was transported to the laboratory for processing.

First, the blood was centrifuged to separate it into its constituent parts. The gold, platelet rich plasma (PRP) top layer was removed and 220µl of 1M $CaCl_2$ was added. After 1 hour in a 37^0C water bath, the platelets were fully aggregated and the purified serum was decanted into labelled microcentrifuge tubes for storage at -80^0C.

Next, the leukocytes were separated from erythrocytes by Dextran sedimentation. 6ml warmed Dextran (GE Healthcare, UK) was added to the remaining cell layer, made up to 50ml with warm NaCl and mixed. This was left to stand for 20-30 minutes, during which time the erythrocytes sedimented. The upper layer containing the cells of interest could then be transferred to a fresh 50 ml tube, which was filled to 50ml with NaCl and centrifuged, in order to isolate the cells.

The PBMCs were separated from other leucocytes using a Percoll gradient. The Percoll stock solution was diluted with phosphate buffered saline without Ca^{2+}/Mg^{2+} (PBS) to form three different solutions of 81%, 68% and 55% concentration.

The gradient: 3ml of 81% Percoll was added to a 15ml Falcon tube. In order to maintain distinct layers, 3ml of 68% Percoll was slowly poured along the inside of the angled tube. The final upper layer was formed by resuspending the centrifuged cells in 3ml of 55% Percoll. This solution was then layered on top of the 68% Percoll layers as before. The gradient was then centrifuged for 20 minutes to separate the cells by mass (figure 2- 2).



mononuclear cells (55%/ 68% interface)

granulocytes (68%/ 81% interface)

residual erythrocytes pellet at the bottom

Figure 2- 2 End result after separation of cells by Percoll gradient

The mononuclear cells were then removed and washed twice in PBS to remove any remaining Percoll. The cells were resuspended in 10ml PBS and counted on a haemocytometer. The cell solution was then divided into the optimum numbers of cells (as determined by previous experiments) for downstream use. 3 tubes of $4x10^6$ cells were apportioned for RNA analysis and 5 $x10^6$ for protein extraction. Any remaining cells were pelleted, then resuspended in 2ml of freezing solution (10% dimethyl sulphoxide {DMSO} and 90% foetal calf serum {FCS}), before being rapidly frozen in a cryofreezer and stored at -80^0C.

To confirm the presence of the required monocytes/ macrophages in the isolated cell samples, immunohistochemistry and flow cytometry was performed on a random selection of samples.

2.3.1.2 Method for cytospin

Purified PBMCs were visualised directly to confirm population type. A solution of $1x10^5$ cells/ml was transferred onto four slides by performing a cytospin. The slides were fixed in methanol. Two of the slides were stained by dipping into Quick-Diff red dye (Reastain, Reagena, Finland) for 1 minute, washing and then Quick-Diff blue dye for another minute. After rinsing the slides, microscope examination verified the population. The other two slides were reserved for HO-1 and CD68 staining (a monocyte/ macrophage surface marker).

a) **b)**

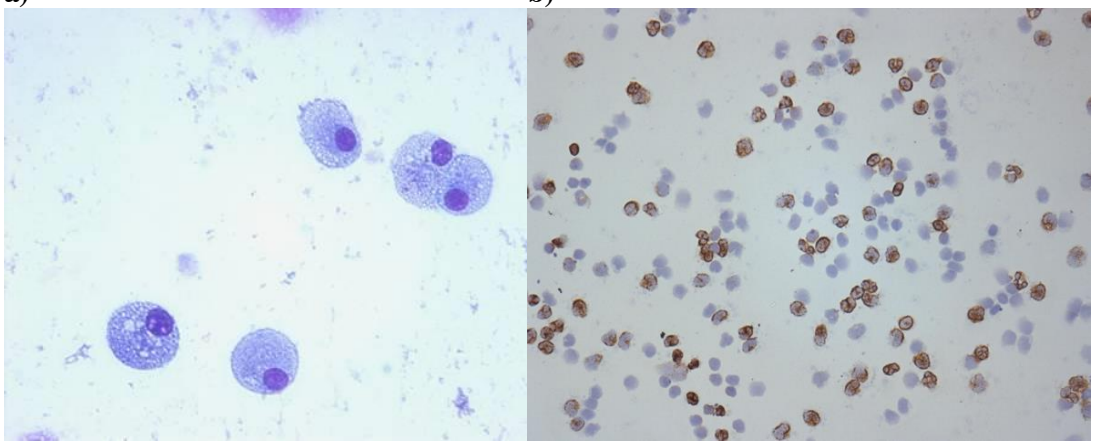


Figure 2- 3 Cytospin images
a) Quick diff stain of isolated PBMCs with macrophages (x40 magnification)
b) PBMC population stained with mouse anti-human CD68 (Abcam, UK) (x10 magnification)

2.3.1.3 Method for Flow cytometry

Small samples of the PBMC and the neutrophil populations were analysed on the flow cytometer (BD, USA). The results were gated for different cell types to confirm the PBMC population.

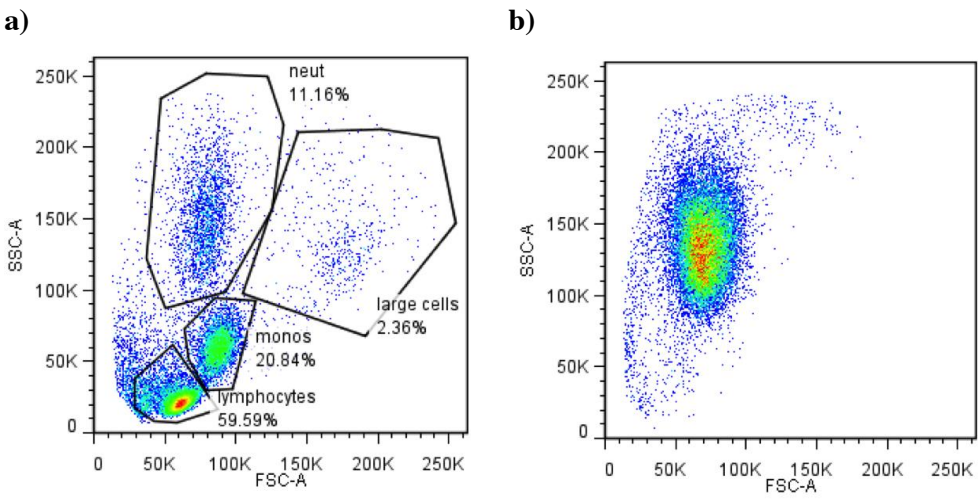


Figure 2- 4 FACS images showing cell population
a) PBMC population
b) Neutrophil population

2.3.1.4 Method for RNA isolation and cDNA synthesis

The Bioline ISOLATE II RNA Mini Kit (Bioline, UK) was used and the protocol was followed. Kit solutions are highlighted in bold.

Cells from 2.3.1.1 were centrifuged, the supernatant removed and 450µl **lysis buffer** added to each sample. Each sample was then resuspended and incubated at room temperature for 3 minutes. Once homogenised, the sample was stored at -20^{0}C until later batch RNA extraction. Figure 2- 5 shows the extraction steps.

Process	Purpose
Cells mixed with **lysis buffer** and vortexed (*frozen until required)	To disrupt cell walls, plasma membranes and organelles in presence of guanidinium thiocyanate to deactivate RNases
↓	↓
Lysate filtered through column	To reduce viscosity and clear lysate
↓	↓
70% ethanol added	To adjust RNA binding conditions
↓	↓
RNA bound onto column	To bind RNA to silica membrane in column
↓	↓
silica membrane desalted	To remove any salt that may reduce DNase activity
↓	↓
DNase added	To digest any contaminant genomic DNA
↓	↓
Washed x3	To remove any impurities such as salts, metabolites and cellular components
↓	↓
RNA and DNAse free water added	To extract RNA for downstream analysis

Figure 2- 5 Process for RNA extraction from PBMC

The concentration of RNA in ng/µl was measured at 260nm by spectrophotometer (Nanodrop, ThermoScientific, USA) and recorded.

The integrity of RNA was randomly tested by agarose denaturing gel. 4μg of a random RNA sample was run through a gel stained with Ethidium Bromide to visualise the ribosomal RNA bands. An optimal RNA sample contained distinct bands at 18S and 28S, with the latter twice as intense as the former.

500μg RNA was transformed into cDNA by reverse transcription using the High-capacity Reverse Transcription kit (Applied Biosciences, UK). The master mix for reverse transcription was prepared on ice using the components below (table 2- 1) and multiplied by the number of samples to be analysed (+2).

Table 2- 1 Components for reverse transcription reaction

Component	**Volume (μl)/reaction**
10 x RT buffer	2.0
25 x dNTP (100mM)	0.8
10 x Random primers	2.0
Multiscribe reverse transcriptase	1.0
RNase Inhibitor	1.0
Nuclease free water	3.2
Total per Reaction	**10.0**

10μl of RNA (diluted with RNase and DNase free water if required) was mixed with 10μl master mix. The samples were loaded into the Thermocycler (Biorad, USA) and the programme was run as per protocol in table 2.2.

Table 2- 2 Reverse transcription protocol

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25^0C	37^0C	85^0C	4^0C
Time	10 mins	120 mins	5 mins	∞

The end result cDNA was stored at -80^0C for later batch analysis.

2.3.1.5 Method for quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The cDNA was defrosted on ice and standardised to 100ng in 20µl with nuclease-free H_2O. An independent lab member randomised the diluted samples. Universal PCR TaqMan Master Mix and TaqMan Gene Expression Assays (Life Technologies, UK) were mixed as per protocol instructions and multiplied by number of samples to be analysed (+2).

Table 2- 3 Components for PCR reaction

Component	**Volume (µl)/reaction**
Master Mix	12.5
HO-1-FAM assay	1.0
18s-VIC assay (endogenous control)	1.0
Nuclease free water	5.5
Total per Reaction	**20.0**

5 µl of each sample was added in duplicate to a 96-well plate stored on ice. Reference standards (cDNA from HA treated macrophages, cDNA from placebo treated macrophages and pure RNA) were also loaded and two wells were left blank.

20µl master mix was added per well. The plate was covered with an adhesive seal and centrifuged before being read on an ABI 7900 PCR machine in University of Edinburgh SURF facility using the "Absolute qualification" standard programme. Once the baseline and threshold values had been defined, the 18s and HO-1 C_T values were saved and exported. If identical replicates had a C_T standard deviation >0.3, the accuracy of the data was questionable and the PCR was repeated.
The mean C_T values for each sample were added to the database where the following calculations were performed.

Delta (**Δ)** C_T was calculated for each sample by normalising HO-1 to the control gene 18s:

$$\mathbf{\Delta C_T = C_T.HO\text{-}1 - C_T.18s}$$

The $\Delta\Delta C_T$ value for each time point (*time x*) including baseline was calculated by the equation:

$$\mathbf{\Delta\Delta C_T = (C_T.HO\text{-}1 - CT.18s).}\boldsymbol{time\ x}\mathbf{ - (C_T.\ HO\text{-}1 - CT.18s).}\boldsymbol{\ baseline}$$

$\Delta\Delta C_T$ for baseline should always be 0.

The fold change in HO-1, normalised to 18s and relative to the expression at baseline, was calculated for each sample (including baseline) using the equation below:

$$\mathbf{amount\ of\ HO\text{-}1 = 2^{-\Delta\Delta C_T}}$$

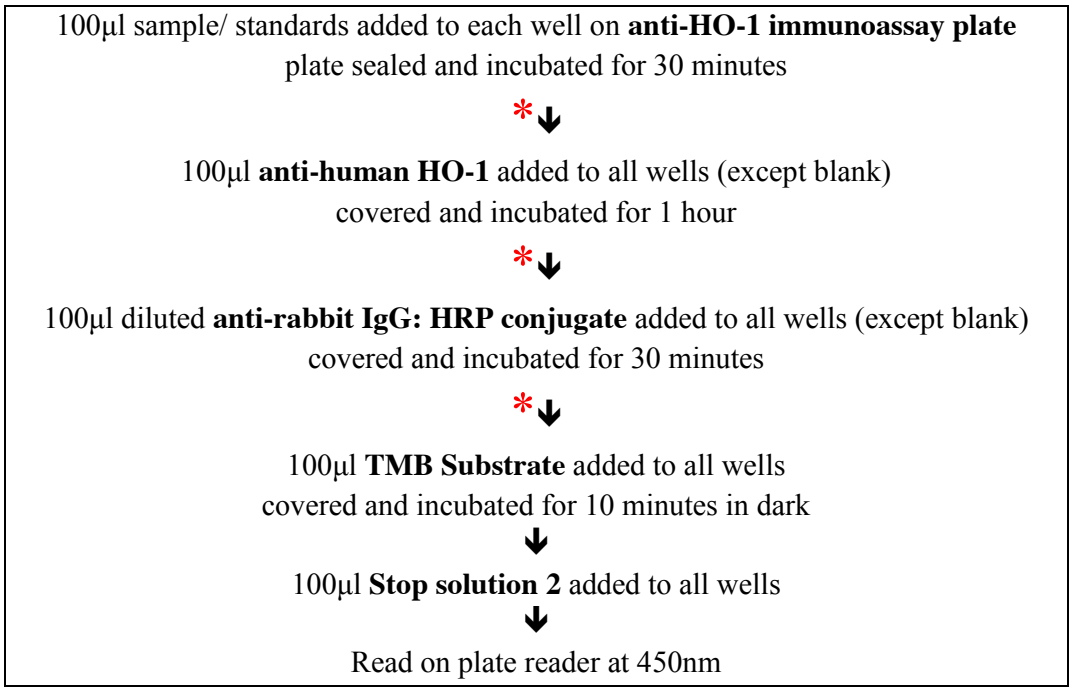
Using this calculation, the value of the mean fold change at baseline should be 1. If this was not the case, the calculations were highlighted and rechecked. The equation $2^{-\Delta\Delta C_T}$ ensured the exponential raw data (C_T) derived from the log-linear plot of PCR signal versus the cycle number was converted to a linear form. This ensures that the mean fold change in gene expression can be compared by standard statistical tests (Livak and Schmittgen 2001).

2.3.1.6 Method for HO-1 protein ELISA

Cells identified for protein extraction in 2.3.1.1 were centrifuged and the supernatant was removed. Cells were resuspended and then lysed. The lysis solution was made by adding 1 tablet of protease and phosphatase inhibitors (Sigma, USA) to 10ml RIPA buffer (Sigma, USA). The appropriate volume (1ml per 0.5 to 5 $x10^7$ cells) was added to the cell pellet and vortexed. This was incubated in the fridge for 5 minutes and then frozen for storage at -80^0C for future use.

Samples were analysed blind in batch. First, the protein concentration of each sample was determined using a Biorad protein assay. The standard curve used BSA (Bovine serum albumin) protein standard in concentrations of 2, 1, 0.5, 0.25, 0.125, 0 mg/ml). 5µl of each sample and standard was loaded (in triplicate for sample and duplicate for standard) onto a 96 well plate. 25µl of solution A and 200µl solution B was added and the plates were covered and developed on a shaking platform for 15 minutes. The plate was read at 750nm. The standard curve was calculated ensuring that regression analysis showed R=close to 1. The concentration of the protein lysate was documented.

The HO-1 Human EIA kit (ENZO, USA) was used as per protocol at room temperature and kit solutions are highlighted in bold. The lysate concentration was standardised to 1mg/ml using **sample diluent** if required to ensure a total volume of 250µl. The 6-point standard curve was created with **HO-1 standard** diluted with **sample diluent** from 25ng/ml to 0.78ng/ml.



*Wash step; six times with **wash buffer**

Figure 2- 6 Process for HO-1 ELISA

The mean of the duplicate absorbance measurements for sample and standards were calculated. The mean blank reading was subtracted from all readings. The standard curve was calculated and the sample concentrations were extrapolated from it. The sample HO-1 concentrations were calculated by multiplying by the dilution factor. The data was added to the database.

2.3.1.7 Method for Western Blot

The methods used to detect HO-1 protein by Western Blot are described below. Unfortunately, the preliminary results were inconsistent and difficult to quantify. The different SOP amendments tried are listed below (table 2- 5) but time restraints prevented further modification. Other researchers investigating PBMCs extracted from human whole blood echoed this experience. Therefore the Western Blot method was rejected and the assay technique described above was used but the methods are included for completeness. All steps were completed at room temperature.

Table 2- 4 Process for making buffers

Running buffer (Tris-HEPES-SDS) **makes 10x solution** • Tris base (MW= 121); 121g • HEPES (MW= 238); 238g • SDS (MW=288); 10g Dissolve in 1L ultrapure water on heating plate	**Transfer buffer (glycine)** **makes 10x solution** • Tris base; 30.3g • Glycine; 144g Dissolve in 1L ultrapure water
Working running buffer **1x solution** • 100ml 10x running buffer • 900ml H_2O	***Working transfer buffer*** **1x solution** • 100ml 10x glycine transfer buffer • 200ml methanol • 700ml pure H_20
Tris buffered saline (TBS) **10x solution** • Tris; 24.2g • NaCl; 80g Dissolved in 800ml ultrapure water Standardise to pH 7.6 by adding HCl Add ultrapure water to final volume of 1L	**TBST** • 100ml TBS x10 • 900ml ultrapure H_20 • 1ml Tween20

2.3.1.7.1 *Sample loading*

The proteins extracted as described in 2.3.1.6 were stored in RIPA buffer. The volume of protein was known and standardised.

A solution of β-mercaptoethanol and sodium dodecyl sulphate (SDS) was made to ratio of 50µl:1ml. 35µl of each sample-loading solution was made of 21µl protein in RIPA, 7µl SDS and 7µl H_2O.

Each sample was heated to 95^{o}C in a heating block for 5 minutes to denature the proteins. Each sample was then centrifuged (1 minute at 10,000g) to remove condensation from the lid.

2.3.1.7.2 *Electrophoresis*

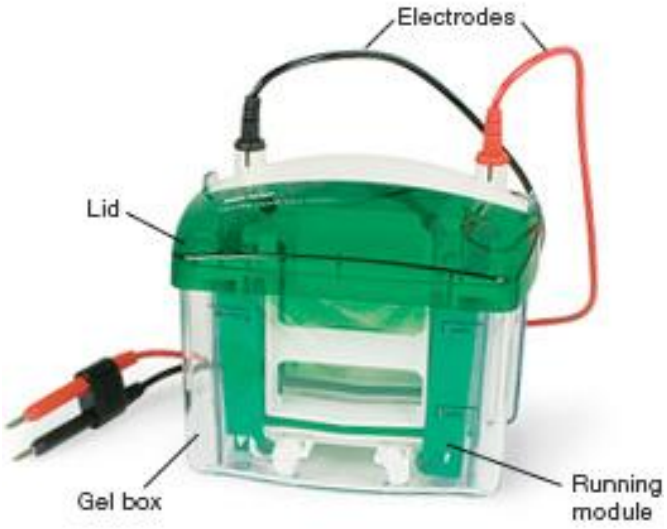


Figure 2- 7 Electrophoresis equipment (from Biorad.com)

The equipment was set-up as in figure 2- 7. The 10-well polyacrylamide gel (Thermo-scientific, UK) was inserted into position. The middle chamber and 3cm of the outer chamber was filled with running buffer to ensure cooling and the sample wells in the gel were rinsed thoroughly with running buffer to remove air bubbles. 7µl of molecular ladder (Biorad, USA) was added to the left hand lane and then, as previously described; 35µl of sample was added to each well using special gel loading tips. Then the remainder of the tank was filled with running buffer. The lid was added and current set to 100V, which forces the proteins to migrate through the gel at different speeds depending on their length and ensures that the proteins are separated by size. Once the samples had nearly reached the bottom of the gel, the machine was switched off and the gel was carefully removed (usually around 45 minutes).

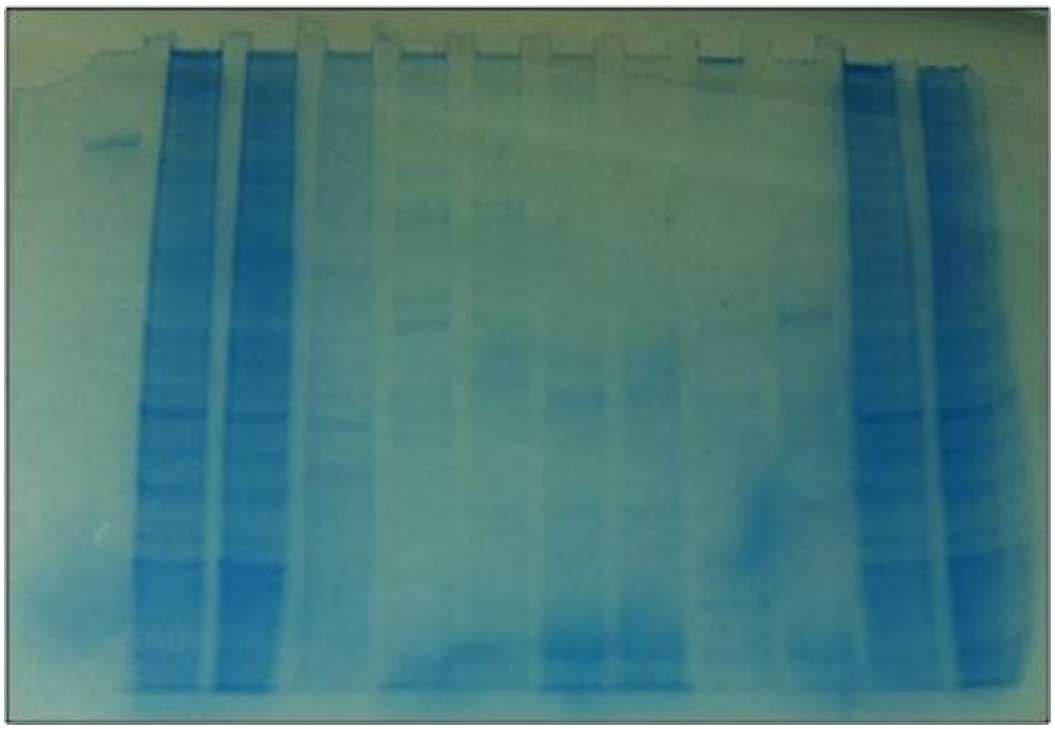


Figure 2- 8 A gel post-electrophoresis showing how the blue samples containing protein move through gel

2.3.1.7.3 *Membrane transfer*

Six pieces of thick filter paper and one piece of membrane (Amersham Hybond ECL, GE Healthcare, USA) were cut to the same size of the gel and all were soaked in transfer buffer, which had been cooled to 4^{o}C. The gel was also soaked in transfer buffer. The semi-dry transfer equipment was set up as in figure 2- 9.

Figure 2- 9 Setting up semi-dry transfer equipment (from Benchfly.com)

The surfaces of the plates were moistened with transfer buffer and the gel "sandwich" was created taking care to remove all bubbles between layers. The order of the "sandwich" was from top;

- anode plate
- filter paper x3
- gel
- membrane
- filter paper x3
- cathode plate

The transfer was then run at 15V for 60 minutes to ensure that the proteins were transferred from the gel to the membrane. When the run was complete, the transfer was confirmed by staining the membrane with Ponceau red dye to detect the protein bands (figure 2- 10). The dye was then washed off in TBST.

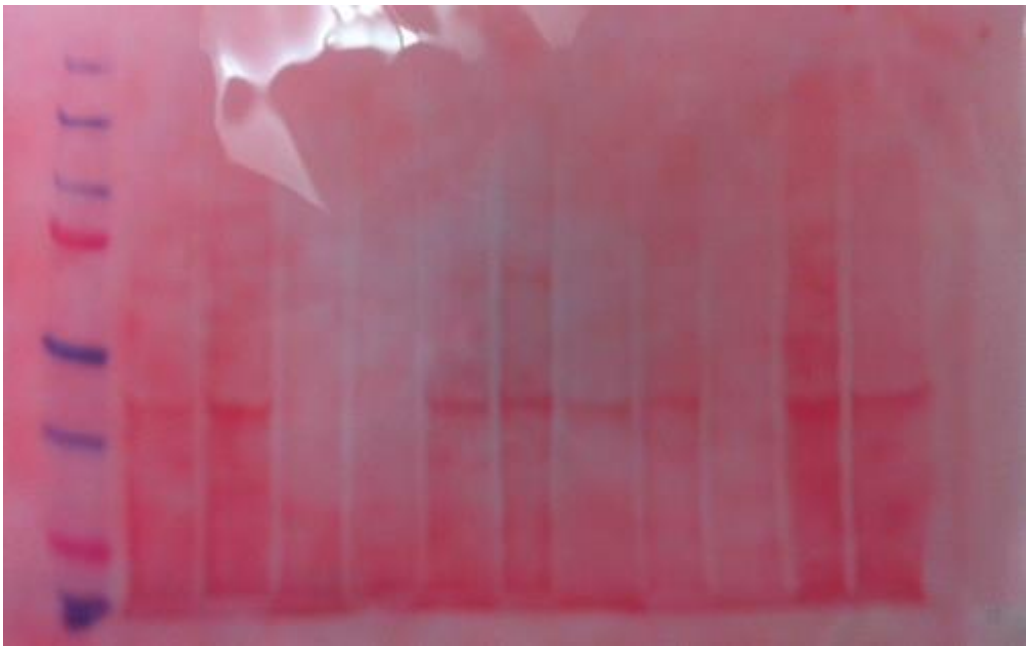


Figure 2- 10 Membrane stained with Ponceau red dye confirming protein transfer. The multi-coloured strip at far left is the molecular ladder, which aids protein detection by marking size of proteins.

2.3.1.7.4 *Protein immunostaining*

The membrane was then blocked to remove non-specific binding. The membrane was carefully added to a Falcon tube and covered with a protein block solution (1g dried skimmed milk in 20ml TBS) and agitated for 20-60 minutes on a roller. The membrane was then rinsed three times with 1x TBST. In order to detect the proteins of interest, the HO-1 and β-actin antibodies (rabbit polyclonal, ENZO) were diluted 1:5000 with antibody diluent (0.5g BSA in 50ml TBS + 25ul TWEEN-20 {TBST}). The membrane was soaked in antibody solution and incubated overnight at 4^{o}C on a roller.

After this, the membrane was washed with TBST five times for five minutes each time. Once washed, 10ml of the secondary antibody solution was added with a 1:2000 solution of goat anti-rabbit horseradish peroxidase (HRP) (ENZO) in the same diluent. This was incubated at room temperature for 35 minutes. The solution was then removed by washing with TBST three times for five minutes each time.

The ECL substrate solution (Pierce, Thermo Scientific, UK) was made as per the instructions and stored in the dark. The membrane was covered in ECL substrate and left for 30 seconds. The excess ECL substrate was removed and transported to machine in film cassette to avoid exposure to light.

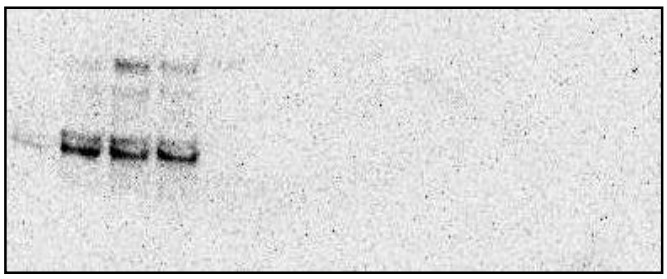
2.3.1.7.5 *Detection of protein bands*

The membrane was photographed on a Versadoc machine at optimum exposure. The images were stored on USB for later analysis on Photoshop. The membrane was then stored in TBST for reanalysis later if required.

Figure 2- 11 Images of failed Western Blots with amended protocols

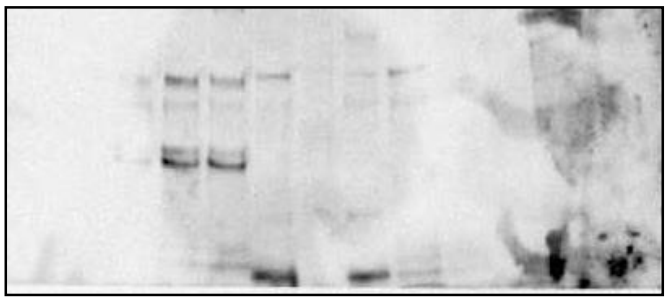
a) 1:5000 primary antibody concentration and standard development techniques. Only 3/ 12 wells are visible

red line= β-actin, blue= HO-1



b) 1:2000 primary antibody concentration and standard development techniques

No discernible difference

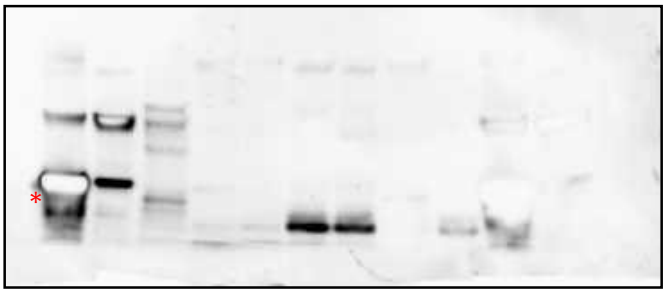


c) 1:2000 primary antibody concentration with super strength developer

More protein detected but sample detection was inconsistent

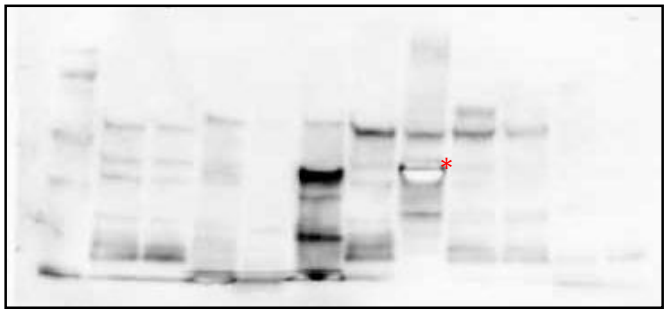
d) Repeated process using previously validated samples,

Only 2 wells detected despite same samples present in other wells



e) Samples from (d) tested with super strength developer left on for longer

Protein detection was improved but inconsistent



f) More protein loaded per well (up to 60µg) with 1:2000 antibody concentration, and superstrength developer left on for twice as long.

Best images but inconsistent results and membrane burnt by long exposure to developing chemicals *.

Table 2- 5 Modifications to protocol to maximise and standardise Western Blot results

Potential explanation for poor results	**Attempted solution**
Insufficient protein in each sample	**Load more protein into each well.** Made a 40µl solution: 31µl protein in RIPA, 8µl SDS, 1µl H_2O
Variation in materials	**Standardise conditions with pre-formed gels and compatible pre-mixed solutions** (from Thermo-scientific, UK)
Poor blocking causing excessive background	**Used BSA instead of milk to block**
Insufficient transfer from sample to gel or gel to membrane	**Experiment with a variety of timings** Gel and membrane transfer were run for varying times after discussion with lab members.
Insufficient antibody to detect protein	**Increased concentration primary and secondary antibody**
Insufficient or insensitive detection	**Use of super-concentrated ECL substrate detection solutions**
Versadoc digital machine not sensitive enough	**Membranes developed using photo paper technique and dark room**

2.3.2 *Renal tissue analysis*

2.3.2.1 Method for renal tissue biopsy

The baseline biopsy sample was taken during the back table examination of the renal graft. A variety of surgeons performed the procedure in a standardised manner consisting of two samples from the superior renal pole taken with a 16g core biopsy gun. One sample was put immediately into formaldehyde and the other into saline for fresh frozen analysis. Both samples were processed in a standard manner by the Pathology Department in Royal Infirmary of Edinburgh (RIE). Three extra slides were produced from the formaldehyde sample for HOT study analysis. A pathologist (or the author if out of hours) divided the fresh sample into a section for standard pathology tests and a section for HOT analysis, which was immediately frozen in solid CO_2. This tissue was transferred to the -80^0C freezer for long-term storage.

The same sample handling method was followed for the day 5 protocol biopsy. A Consultant Radiologist in the RIE Radiology Department performed the biopsy under ultrasound guidance.

2.3.2.2 Method for RNA isolation

Frozen renal tissue was homogenised using rod and needle. As per the ISOLATE II RNA Mini kit protocol (Bioline, UK), the lysis solution was added and vortexed. From this point, RNA extraction was the same as 2.3.1.4.

2.3.2.3 Method for protein analysis

The initial plan was to analyse renal tissue for HO-1 protein by Western Blot. However, this was not possible due to the small size of the biopsy specimens. Sample quantification experiments showed that >5mm core sample tissue was required for reproducible and accurate protein results. This was only achieved in 3/37 back table samples. Therefore, immunohistochemistry (IHC) was used to quantify HO-1 protein in renal samples.

2.3.2.4 Method for single stain immunohistochemistry (IHC)

Renal tissue samples were stained for HO-1 using rabbit anti-HO-1 antibody (Abcam, USA) with human tonsillar tissue used as positive control. After optimisation in the laboratory, the participant slides were stained as a single batch in the SURF facility. For each slide, all of the tissue was photographed at x20 magnification with no overlap. The HO-1 positive area was calculated using computer image analysis (Image J, http://imagej.nih.gov/ij). For each Image J field, the positive areas (stained brown) were identified and the corresponding number of pixels was recorded. The total area of tissue was also quantified using Image J and the percentage of HO-1 staining to total tissue area was determined.

2.3.2.5 Method for dual stain immunoflurescence (IF)

The blinded slides were stained by the SURF facility at University of Edinburgh using rabbit anti- human HO-1 antibody (Abcam) and anti-CD68 antibody (Abcam). Between four and eight non-overlapping fields at x20 power were photographed on the LSM 710 confocal microscope.

The total number of CD68 positive cells per field was counted. The number of dual stained cells in each field was counted and this was expressed as a percentage of the total CD68 cells per field. Then four fields were blindly chosen and their results were entered into the database for subsequent statistical analysis.

2.3.2.6 Method for histological assessment

Histological analysis was performed blind by Dr C Bellamy (Consultant Renal Pathologist) using a 13-point system to identify indicators of cell injury.

The cumulative score divided samples into 5 groups.

0= no evidence of injury

1= mild injury

2= minimal injury

3= moderate injury

4= severe injury

This score was added to the database for subsequent analysis.

2.3.3 *Urine analysis*

The biomarkers NGAL and KIM-1 were measured in urine samples.

2.3.3.1 Urine collection

Participant urine was collected from catheter bags and apportioned into 5 x 2ml microcentrifuge tubes, labelled with participant number and frozen in -80^{0}C freezer.

2.3.3.2 Method for biomarker assay (KIM-1 and NGAL)

The two biomarkers were analysed using DuoSet human Lipocalin-2/NGAL ELISA and DuoSet human TIM-1/KIM-1 ELISA (both R&D Systems, USA). The kit protocols were followed and all was performed at room temperature unless stated. Kit solutions are highlighted in bold.

Samples were analysed in duplicate and diluted to optimum concentration with reagent diluent (R&D Systems). The standard curve used a 7-point dilution of **recombinant human KIM-1/ NGAL** made with reagent diluent with a max concentration of 2ng/ml (KIM-1) and 5ng/ml (NGAL).

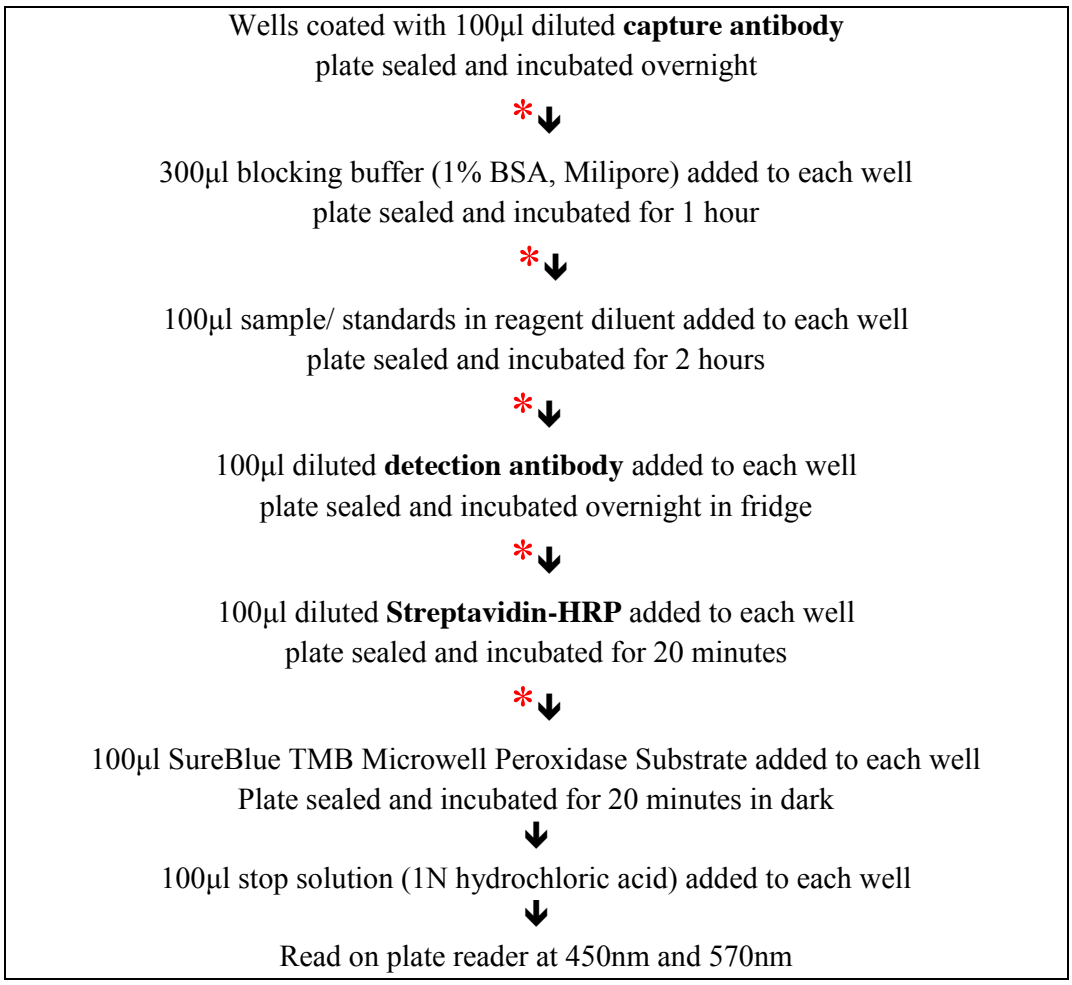


Figure 2- 12 Process for urine biomarker ELISA

The mean of the duplicate absorbance readings for sample and standards were calculated (450nm). The mean blank reading (570nm) was subtracted from all mean standards and samples. The standard curve was calculated and the sample concentrations were extrapolated from it. The NGAL/ KIM-1 concentration was determined by multiplying by the dilution factor. The results were added to the database.

2.3.3.3 Method for creatinine measurement

The QMRI lab measured the creatinine concentration for each urine sample to allow the biomarkers to be accurately compared taking into account differences in urine concentration.

2.3.4 *HO-1 genotype*

A sample of DNA was extracted from the baseline blood sample. The recipient genomic DNA was assayed to determine the HO-1 $(GT)_n$ genotype.

2.3.4.1 DNA extraction

The DNeasy Blood and Tissue kit (Qiagen, USA) was used and the protocol was followed. Kit solutions are highlighted in bold. Anti-coagulated blood taken at admission was used.

Process	Purpose
100µl blood, 100µl PBS and 20µl **proteinase K** mixed	To lyse cells
↓	↓
200µl **buffer AL** added	To optimise DNA-binding conditions
↓*	↓
incubated 56^0C for 10 minutes	To improve cell lysis
↓	↓
200µl ethanol added	To aid binding
↓*	↓
Add mixture to DNeasy spin column and centrifuge	To bind DNA to silica membrane and contaminants pass through
↓	↓
Washed x2 with **buffer AW1** & **AW2**	To remove any impurities such as salts, metabolites and cellular components
↓	↓
50µl **Buffer AE** added, incubated for 1 minute and centrifuged	To elute DNA for downstream analysis

Figure 2- 13 Process for DNA extraction *vortex

The quality and quantity of DNA was determined spectrophotometrically and recorded.

2.3.4.2 Method for genotyping

Fragment length analysis involved amplification of the DNA by PCR using the TaqMan PCR kit (Life Technologies, UK) and size analysis using capillary electrophoresis (Courtney, McNamee et al. 2007).

The components listed in table 2- 6 were mixed in a RNA-and DNA-free microcentrifuge tube and loaded into the Thermocycler machine (Biorad, USA.) The primers (forward:5'-AGA GCC TGC AGC TTC TCA GA-3'; reverse 5'- ACA AAG TCT GGC CAT AGG AC-3') were modified with a 5'-FAM label on the forward primer (Invitrogen, Carlsbad, CA) (Exner, Bohmig et al. 2004).

Table 2- 6 Components for PCR reaction

Component	**Volume (µl)/reaction**
Template DNA	2.0
5µM forward primer	1.0
5µM reverse primer	1.0
2µM dNTP	1.0
10x buffer	1.0
Taq polymerase	0.125
Double distilled water	3.875
Total per reaction	**10.0**

Table 2- 7 Programme settings for the PCR reaction

	Step 1	Step 2 (x 40 cycles)	Step 3 (x 41 cycles)	Step 4 (x 41 cycles)	Step 5
Temperature	95^0C	93^0C	51^0C	72^0C	72^0C
Time	12 mins	40 seconds	40 seconds	60 seconds	15 mins

The PCR product was diluted to the optimum concentration with triple distilled H_2O. 1µl of the diluted PCR product and 9µL of internal standard was added to each well of the microtitre plate and mixed by pipetting. The internal standard consisted of 10µl GeneScan 500LIZ size standard in 1ml HiDi formamide (both from Applied Biosystems, USA). Analysis of the samples was performed on an ABI3730 capillary sequencing machine at GenePool, a Sequencing and Bioinformatics company at the University of Edinburgh. The results were presented as electropherograms, which were visualised and interpreted on Peak Scanner software (Applied Biosystems). There was one graph for each participant sample.

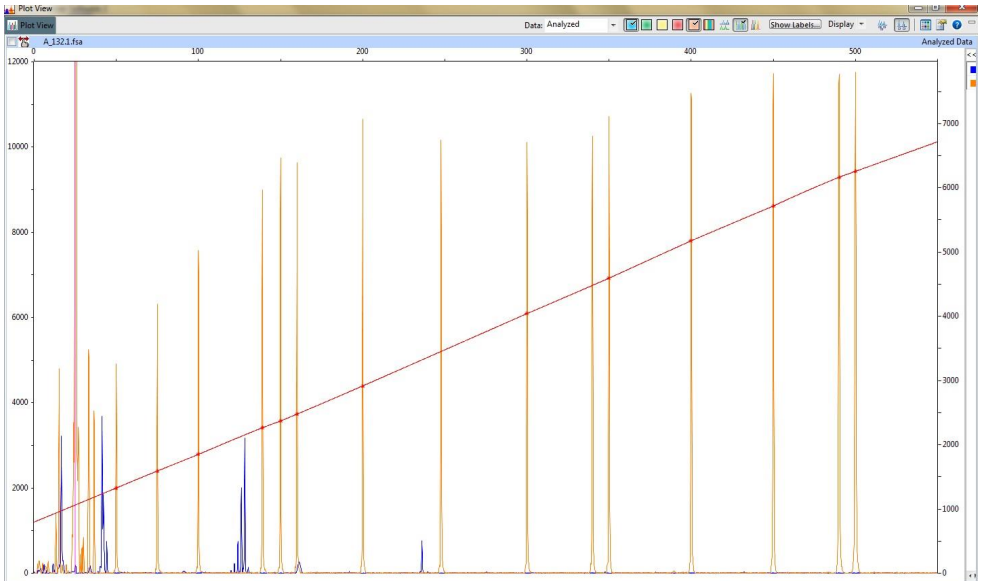


Figure 2- 14 Sample electropherogram; blue peaks are sample product and orange peaks are size standard

2.3.4.3 Analysing electrophoresis results

In the electropherogram, the y-axis is relative fluorescent units (RFU) and the products in the sample are in base pairs along the x-axis separated by size with smallest on left and largest on right. Each graph was analysed to determine the size of the allele of interest (the HO-1 promoter allele). Most graphs had a number of blue peaks but in each sample, there should be only one (if the individual was homozygous) or two peaks (if heterozygous) that represent the true allele. This was occasionally complicated by background noise, which had to be excluded. The product size of interest was calculated as between 87 and 159 base pairs (bp) based on the promoter sequence published by Shibahara (Shibahara, Sato et al. 1989) and so any blue peaks outside this range could be ignored as noise. Any peaks that fitted the basic allele morphology; a peak with a narrow base and a high RFU, were examined more closely. A homozygous allele had a single, tall peak compared to a heterozygous individual whose graph had two smaller peaks side by side. The size discrepancy is due to the fact that homozygous alleles have all the PCR product accumulating at one point, resulting in a higher RFU. The size of the allele (in bp) was noted (usually between 103 and 128bp) and the size of the GT dinucleotide microsatellite was calculated by subtracting the size of the primers (known to be 67bp) from the size of the allele. The number of GT repeats was then calculated by dividing by 2. This process was repeated for the second peak if heterozygous.

A participant's HO-1 genotype could then be determined. The short allele was classed as <25 GT repeats, the long allele $\geq$25 repeats. All participants were then characterised as either homozygous (SS or SL) or heterozygous (SL).

2.4 Statistical analysis

Independent blinded statisticians from the University of Edinburgh analysed the HOT study database using the pre-determined endpoints to generate the study report.

PBMC and renal HO-1 expression data, urine biomarkers and infiltrating macrophage data were not normally distributed; therefore a non-parametric Mann-Whitney statistical test was used for these outcomes.

For HO-1 upregulation in renal tissue, a Fisher's test was used to compare percentages between the groups. A comparison of proportions test was used to compare rates of DGF between groups.

A chi^2 test was used to look for any evidence of difference in renal tissue injury scores between groups.

Results are presented as median (IQR), mean $\pm$SEM or and % values (95% confidence intervals) as appropriate. Statistical analysis was performed on SDSS.

Results are considered significant at $p<0.05$.

Chapter 3.

Results

3.1 HOT Study results

3.1.1 *HOT study recruitment*

The trial began in January 2012 and continued until 40 patients had been randomised and received the infusion in May 2013 (figure 3- 1).

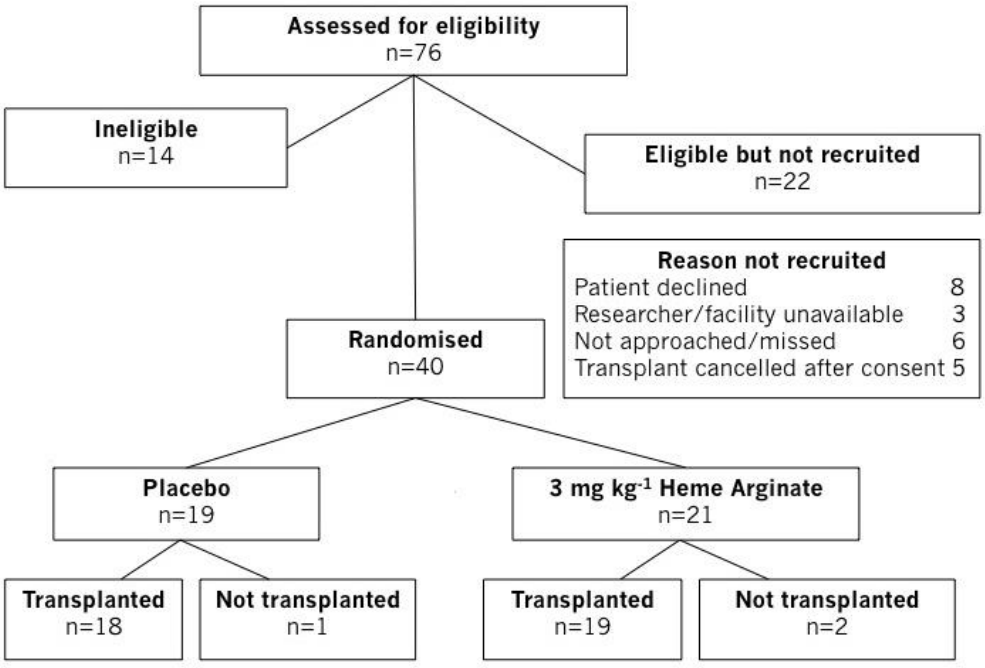


Figure 3- 1 Flow chart of HOT study recruitment

The **reasons for ineligibility** are included in table 3-1.

Table 3-1 Reasons for patient ineligibility

Reason for ineligibility	**Numbers**
Unable to receive standard immunosuppression	5
>2 previous transplants	3
Anti-coagulation/ 2 anti-platelet medication	6
Total	**14**

The renal grafts for the **three non-transplanted participants** were suboptimal at back-table examination and therefore, the transplants were cancelled after the infusions had been given. The HOT study protocol stated that once the participant had been randomised and given a trial number, it could not be reassigned. As per protocol, the patient was observed for the remainder of their time in hospital and their baseline data was recorded in the database.

All participants who received a transplant provided a blood sample at 24 hours for primary outcome analysis. Due to withdrawal of consent or problems with venous access, not all participants gave all blood samples. One participant in each group refused the second infusion and all subsequent blood samples. The two participants did not withdraw their consent for collection of their clinical data and so remained in the trial (figure 3- 2). There were no other withdrawals.

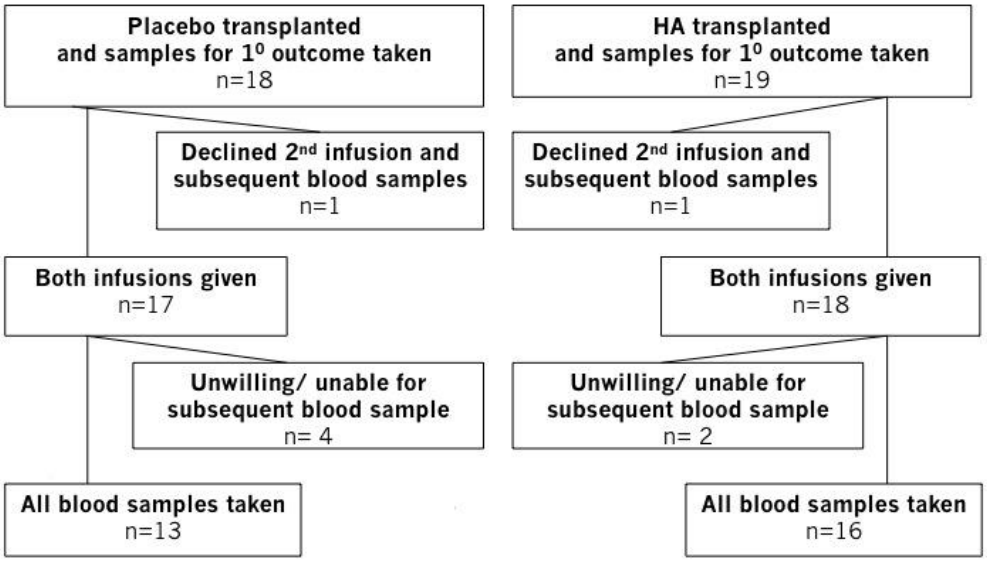


Figure 3- 2 Flow chart with breakdown statistics of study blood samples and infusions

Five participants in the placebo group and three participants in the HA group declined the day 5 biopsy and two in each group were not considered fit or safe for biopsy. As a result, there were 10 paired renal samples in the placebo group and 13 in the HA group (figure 3- 3).

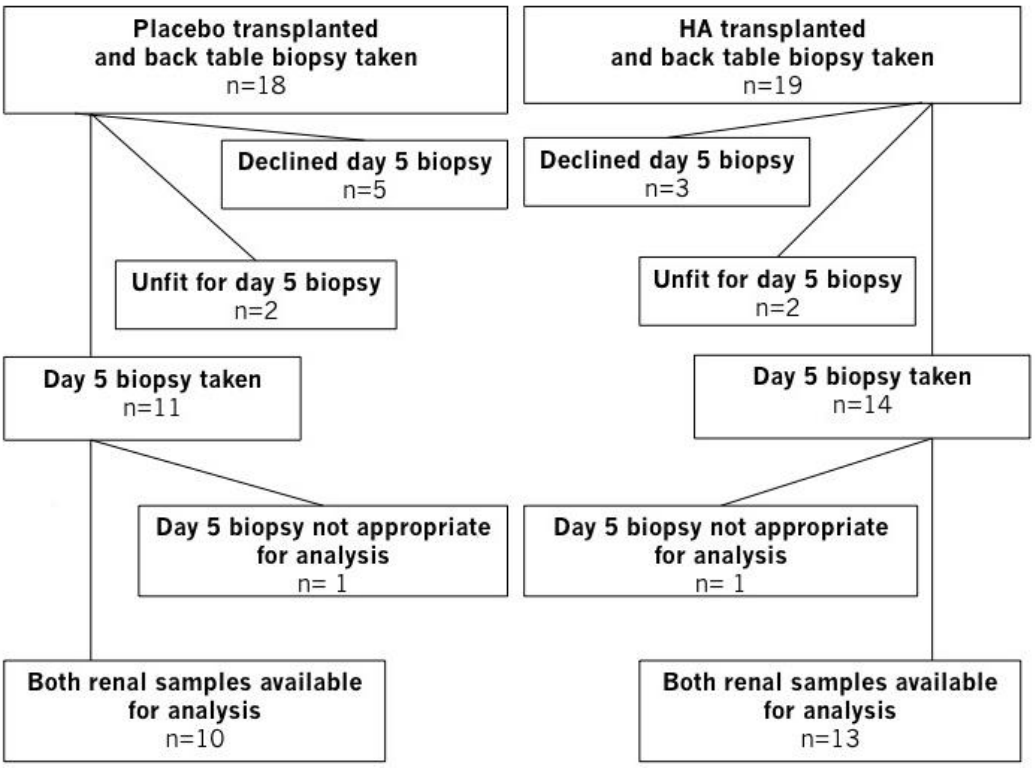


Figure 3- 3 Flow chart with breakdown statistics of study biopsies

Recruitment rates varied throughout the study period (figure 3- 4) in keeping with the reported experience of annual variations in transplant activity.

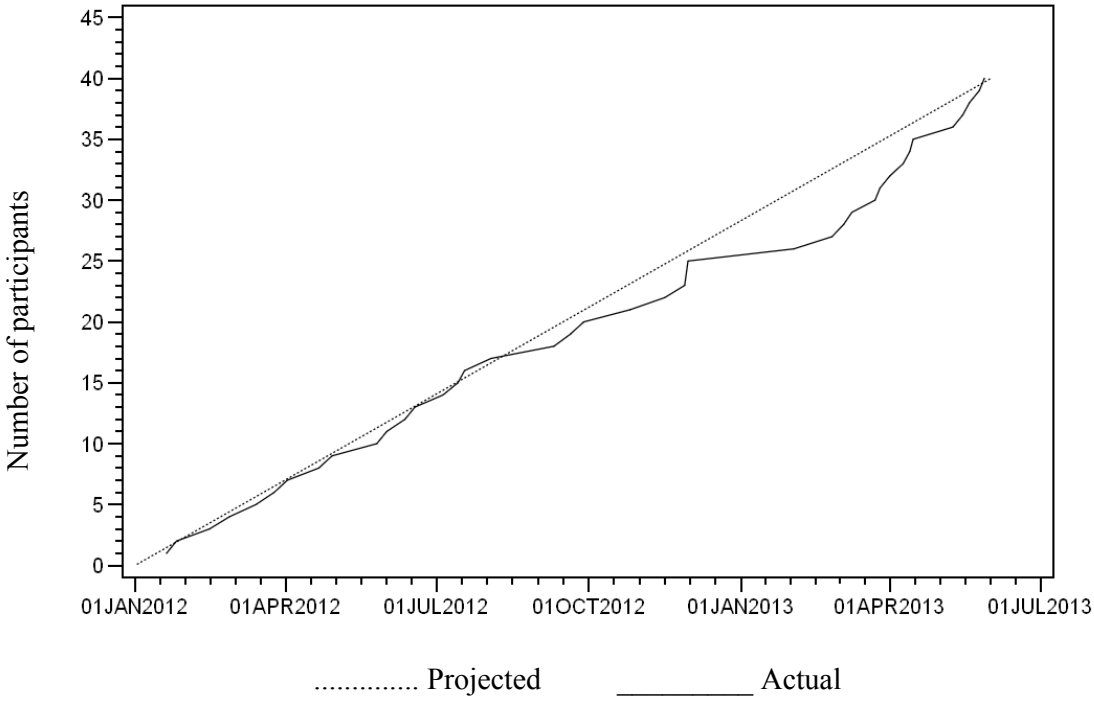


Figure 3- 4 Recruitment rates during study period

3.1.2 *HOT study participants*

The baseline characteristics were similar between the groups.

Table 3- 2 Baseline characteristics of the 37 transplanted patients by treatment group

Characteristic	**Placebo (n=18)**	**HA (n=19)**
Mean age (range)	52.4 (25-79)	52.1 (21-69)
Male (%)	11 (61)	12 (63)
1st transplant (%)	18 (100)	18 (95)
2nd transplant (%)	0	1 (5)
Dialysis patient (%)	17 (94)	18 (95)
Dhx: statin (%)	11 (61)	12 (63)
Dhx; any antiplatelet agent (%)	8 (44)	6 (32)
HO-1 genotype (%)		
SS	2 (11)	0
SL	9 (50)	10 (53)
LL	6 (33)	9 (47)
Mean time from infusion to reperfusion (minutes) [range]	420.6* [39.0- 1586.0]	249.0* [48.0- 803.0]
Donor characteristics		
Mean age (range)	45.2 (21-73)	46.8 (14- 69)
Male (%)	13 (72)	10 (53)
DBD (%)	9 (50)	10 (53)
DCD (%)	9 (50)	9 (48)
ECD[1] (%)	2^ (11)	4^ (21)
Mode of organ perfusion- hypothermic (%)	3 (17)	2 (11)
Mode of organ perfusion- cold static (%)	15 (83)	17 (89)

Mean histology score from day 0 sample[2]	1.8+	2.2+
HLA match 0 HLA-DRB1 mismatch 1 HLA- DRB1 mismatch 2 HLA-DRB1 mismatch	 3 (17) 9 (50) 6 (33)	 1 (5) 13 (69) 5 (27)
Number with virtual crossmatch[3]	12 (67)	15 (79)
Mean CIT in minutes[4] (range)	691.7 (351- 1287)	731.5 (450- 1213)
Mean WIT in minutes[5] (range)	36.2 (28- 61)	37.6 (23- 59)

Mean values are given for continuous variables, while numbers of patients (percentages) are given otherwise.

[1] Extended criteria donor includes donors > 60 years old or >50 years old with two of the following; hypertension, cerebrovascular accident as cause of death, creatinine >1.5 mg/dl (132.6 μmol/L)

[2] Score ranges from 1-4 with 1 as least injured and 4 as most, based on 13-point score of histological features

[3] Virtual crossmatch= in carefully selected patients that are not sensitised it is possible to undertake the transplant without waiting for a full cross match to be performed. This is known as a virtual crossmatch, and results in reduced cold ischaemic times.

[4] CIT= Cold ischaemic time= time from storage in ice at recovery to removal from ice during transplant operation

[5] WIT= Warm ischaemic time (2nd)= time from removal from ice for transplant to reperfusion

* p= 0.50, ^ p= 0.42, +p= 0.35

3.1.3 *HOT study follow-up*

30-day and 90-day follow-up was performed for all participants who received a transplant except the one participant in the placebo group who lost their graft due to a technical problem.

3.1.4 *HOT study safety outcomes*

The emergency unblinding procedure was never required.

No adverse reactions and no deaths occurred during the seven-day trial period.

3.1.4.1 Adverse events

Table 3- 3 Adverse events within 30-day trial period

Adverse event		**Placebo (n=19)**	**HA (n=21)**
ITU admission		1* (6) (pulmonary oedema)	0
Haemorrhage (evidence of hypovolemia requiring transfusion)		1* (6)	2* (11)
Reoperation/ procedure within 7 day trial period. Both events occurred prior to day 5 biopsy		1* (6) (nephrectomy for uncontrollable haemorrhage)	1* (5) (embolectomy for arteriovenous {AV} fistula)
Infection requiring antibiotics		3 (16)	5 (24)
(site recorded below)	LRTI	1	3
	Urine	1	1
	Other	1	1
Systemic Inflammatory Response Syndrome (SIRS)		1 (6)	0
Acute rejection recognised within 7 days		1 (6)	2(11)
AV fistula requiring treatment		0	1* (5)
Graft loss within 7 days		1* (6)	0

Whole numbers (percentage)

*Classified as serious and expanded below

The graft loss was due to technical reasons and they returned to the waiting list.

3.1.4.2 Serious adverse events

There were five serious adverse events during the trial reporting period: three in the placebo and two in the HA group. The sponsors, DMC and MHRA were satisfied with the classification and reporting of the events.

Placebo group;

- One participant had a delayed discharge due to loose stools so the event met the criteria of SAE although it was diagnosed as a side effect of immunosuppression.
- One participant was admitted to ITU as a result of acute respiratory distress, which was initially diagnosed as a chest infection but then they developed pulmonary oedema and atrial fibrillation. They improved within 24 hours and made a good post-op recovery once back on the ward.
- The final participant developed hypotension and was taken to the operating theatre twice for exploration of graft. Unfortunately the team were unable to repair the renal vein and a nephrectomy was required on day 7. This was unrelated to the day 5 protocol biopsy.

HA group (neither was related to the IMP);

- One participant developed hypovolaemic shock on day 5 and a CT scan diagnosed an AV fistula, which was thought to be secondary to the back-table biopsy. They required a blood transfusion (two units packed red cells) and embolectomy with x-ray imaging. The day 5 biopsy had not been taken.
- The other participant required transfusion after a localised haemorrhage when the drain was removed on day 2.

3.2 Effect of HA infusions on PBMCs

In all graphs p value is represented by *.

****= p≤0.0001, *** = p≤0.001, **= p≤0.01, * = p≤0.05.

3.2.1 *HO-1 protein expression*

The primary outcome measure was the change in HO-1 protein expression in the recipient PBMC population from pre-infusion (D0) to 24 hours post-infusion (D1) between the placebo group and the HA group.

HA upregulated HO-1 concentration by 11.1ng/ml (1.0- 37.0), compared with placebo -0.14ng/ml (-0.7- 0.3) (p=<0.0001) (figure 3- 5). One participant did not have a baseline (D0) HO-1 protein measurement so this participant was excluded from the primary outcome analysis (n=36).

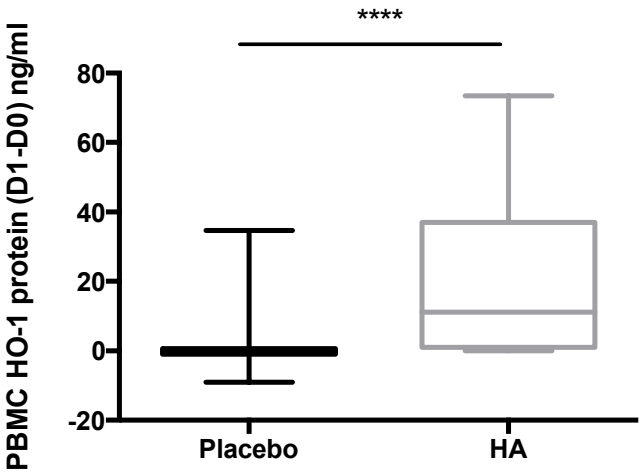


Figure 3- 5 Difference in HO-1 protein expression in PBMC at D1 compared to D0

As expected, there was a peak in HO-1 PBMC protein concentration after each HA infusion (first infusion given immediately after D0 sample and second after D2 sample) (figures 3- 6 and 3- 7).

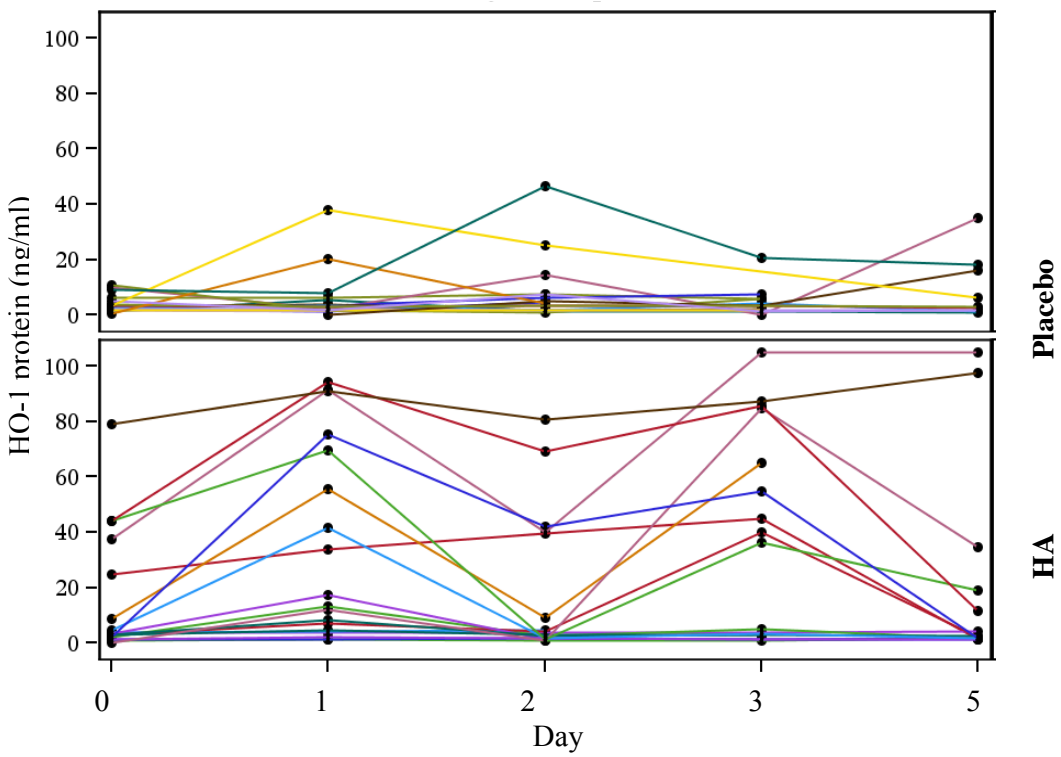


Figure 3- 6 Pattern of PBMC HO-1 protein expression over the six days of the study. Each coloured line represents a different participant.

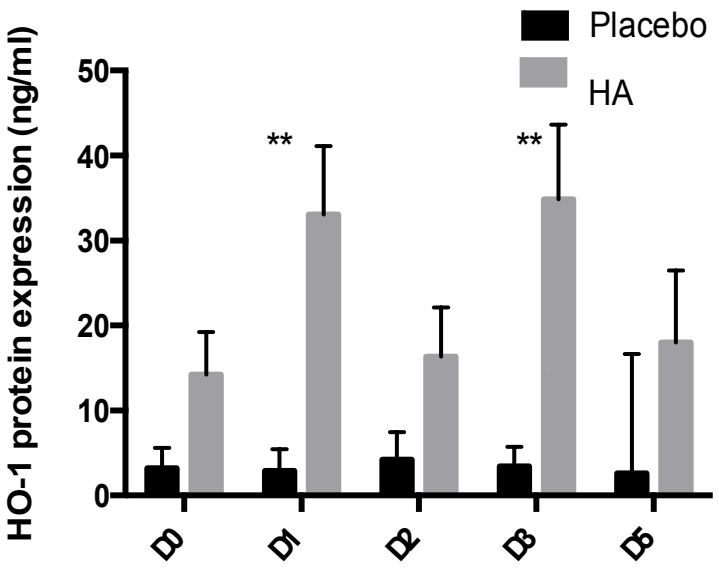


Figure 3- 7 PBMC HO-1 protein expression over the six days of the study

3.2.2 *HO-1 mRNA expression*

The HO-1 upregulation was confirmed by HO-1 mRNA. Delta (Δ) C_T values were calculated for baseline (D0) and day 1(D1). $\Delta\Delta C_T$ was performed to calculate the difference in mRNA upregulation at D1 relative to D0. $2^{-\Delta\Delta C_T}$ calculation was performed to determine the fold increase from D0 to D1.

HA treatment upregulated HO-1 mRNA expression 2.73 fold (1.8- 3.2) compared with placebo 1.41 fold (1.2- 2.2) (p=0.02) (figure 3- 8).

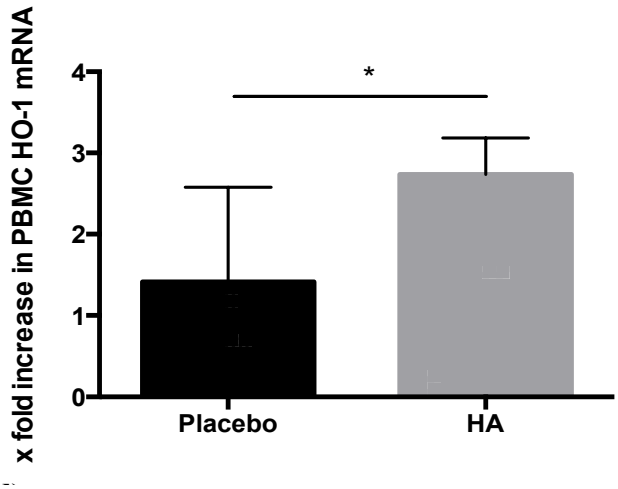


Figure 3- 8 Comparison between HO-1 mRNA upregulation in PBMCs

As for HO-1 protein, the pattern of individual (figure 3- 9) and median (figure 3- 10) HO-1 expression over the 5-day study was characterised. Although there was also a peak in HO-1 mRNA expression after the second infusion, this did not reach significance (figure 3- 10).

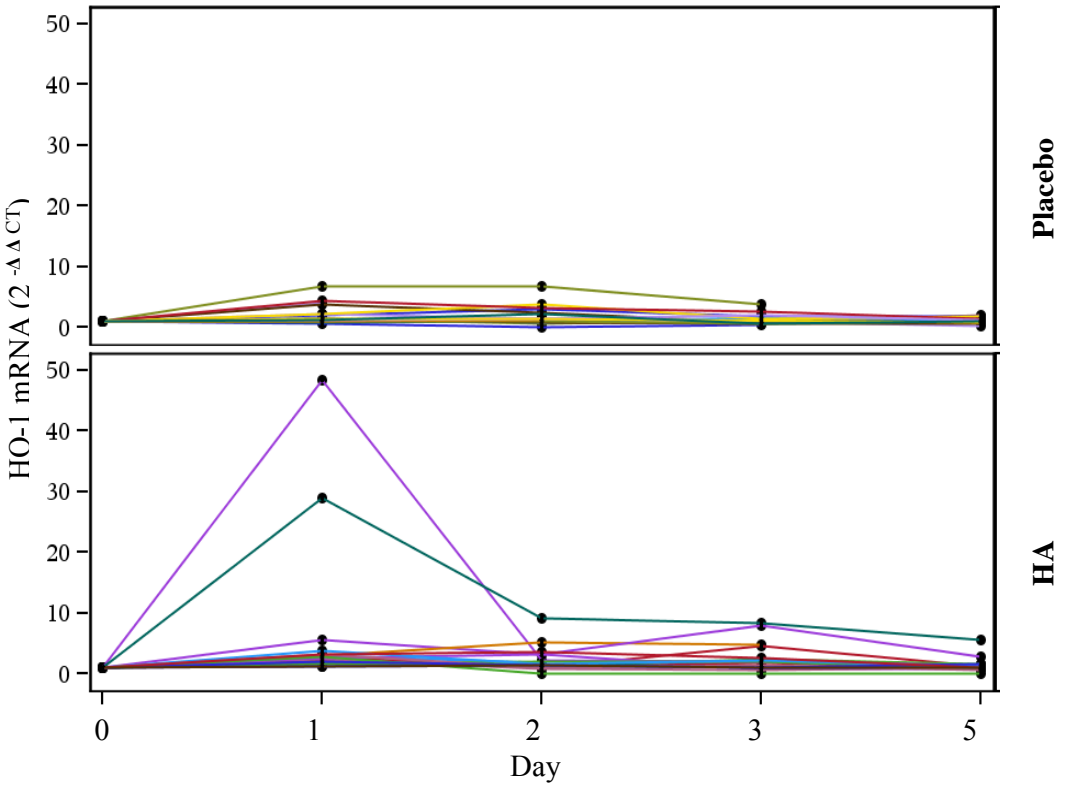


Figure 3- 9 Pattern of PBMC HO-1 mRNA expression over the six days of the study. Each coloured line represents an individual participant.

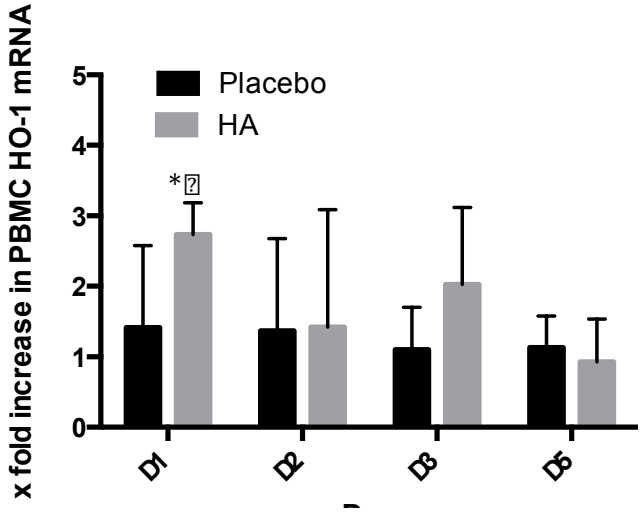


Figure 3- 10 PBMC HO-1 mRNA expression over the six days of the study

3.3 Effect of HA infusions on tissue macrophages

After allowing for declined and insufficient biopsies, there were 9 paired renal samples in the placebo group and 12 in the HA group for comparison.

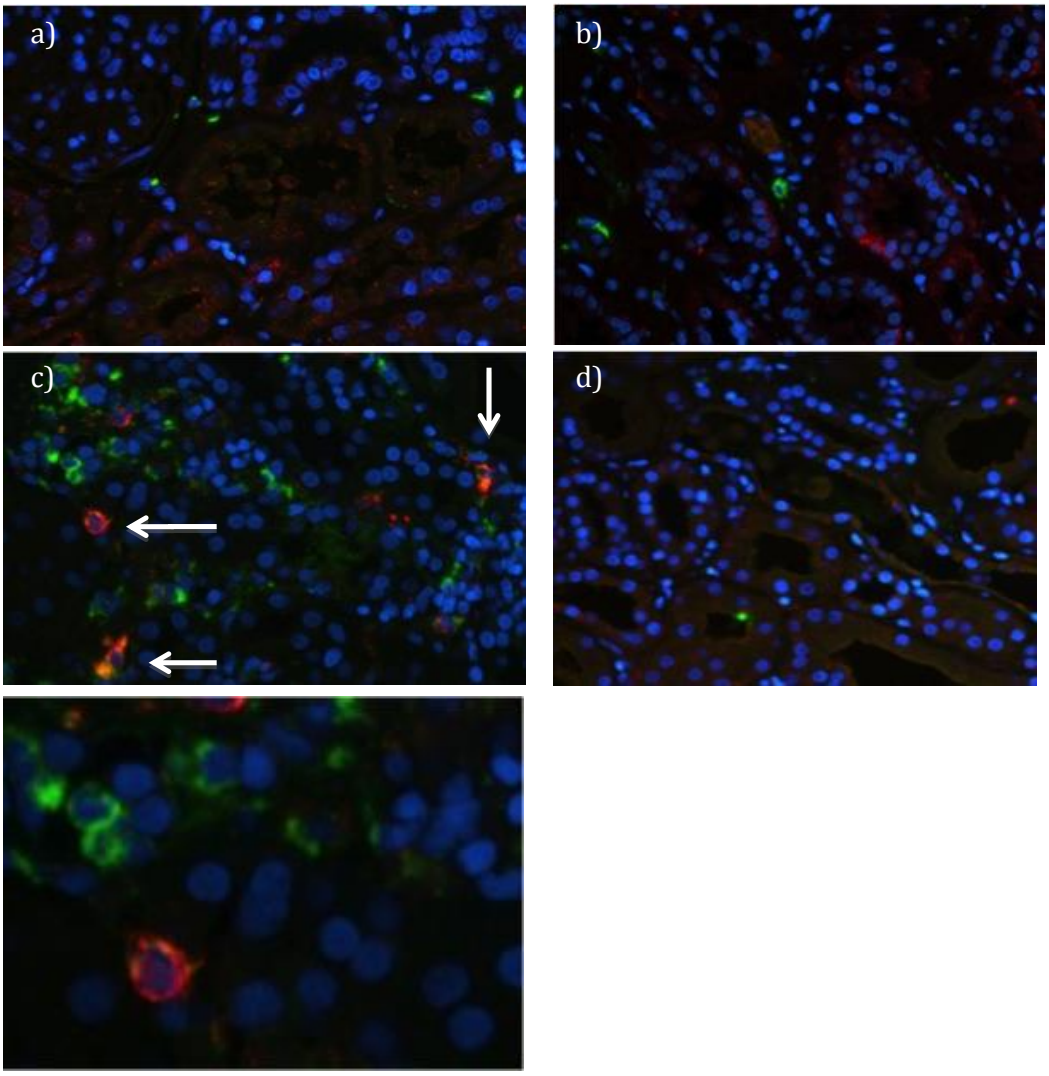


Figure 3- 11 Immunofluorescence images with dual CD68 (green) and HO-1 (red) staining in renal tissue from a) D0 HA, b) D0 placebo, c) D5 HA, d) D5 placebo. Blue= nuclear stain. White arrows highlight dual stained cells (x20 magnification) of which one is enlarged in e).

There was no significant difference in the numbers of CD68 positive cells between treatment groups at either D0 or D5.

At D0, there were 3.50 (1.5- 5.25) CD68 positive cells per high-powered field (HPF) in HA group compared to 3.00 (2.25- 4.25) in placebo group (p=0.956).
At D5, there were 11.0 (4.5- 25.0) cells per HPF after HA compared to 7.38 (4.8- 9.13) after placebo (p=0.13) (figure 3- 12).

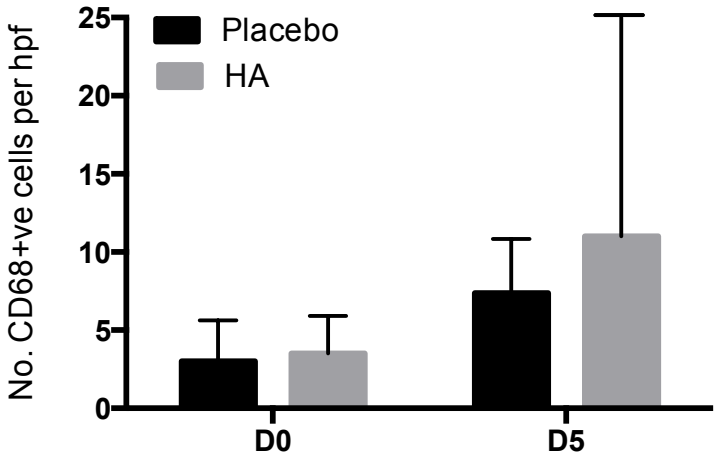


Figure 3- 12 Number of CD68 positive macrophages at D0 and D5

The percentage CD68-positive macrophages expressing HO-1 (so-called dual positive cells) at D0 and D5 were compared between groups. As there were no cells that only stained for HO-1, the percentage of dual stained cells was calculated using the equation:

$$\% \text{ dual stained} = \left(\frac{\text{number of dual positive cells}}{\text{number of CD68 positive cells}} \right) \text{x100}$$

At D0 there were no dual positive cells in either treatment group.

At D5, there were 50.81 cells per HPF (40.0- 59.8) after HA treatment and 22.3 (0-34.8) after placebo. This was significant (p=0.012) (figure 3-13).

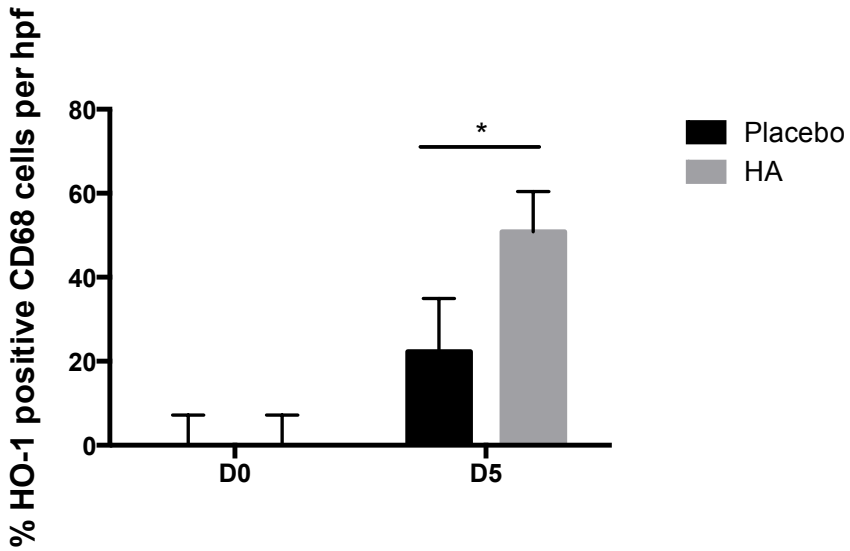


Figure 3- 13 Percentage CD68 positive cells that expressed HO-1 (dual positive) at D0, D5

In addition the difference between the % dual stained cells in each paired samples was calculated and compared between groups;

Difference = % dual stained D5 - % dual stained D0

This was calculated as a 5% $\pm$ 30.6 increase in % dual stained cells in the placebo group vs. a 46.2% $\pm$ 24.0 increase in the HA group, which was statistically significant (p= 0.007).

3.4 Effect of HA infusions on renal tissue

3.4.1 *HO-1 protein expression*

The difference in amount of HO-1 protein expression (as determined by immunohistological staining) between back table (baseline, D0) and day 5 (D5) samples was calculated and then compared between HA and placebo.

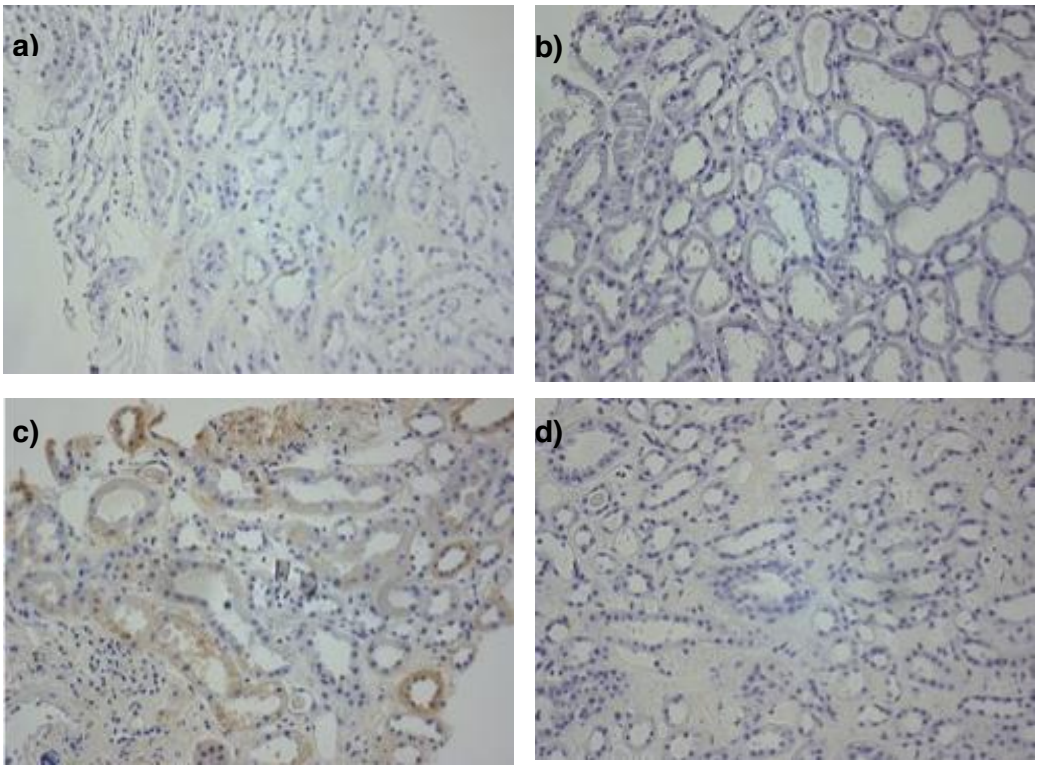


Figure 3- 14 Immunohistological staining for HO-1 (brown) in renal tissue at a) D0 HA, b) D0 placebo, c) D5 HA, d) D5 placebo (x20 magnification)

There were 10 paired samples in the placebo group and 12 paired samples in the HA group suitable for analysis.

The DAB staining (brown) was quantified as a percentage of total renal protein:

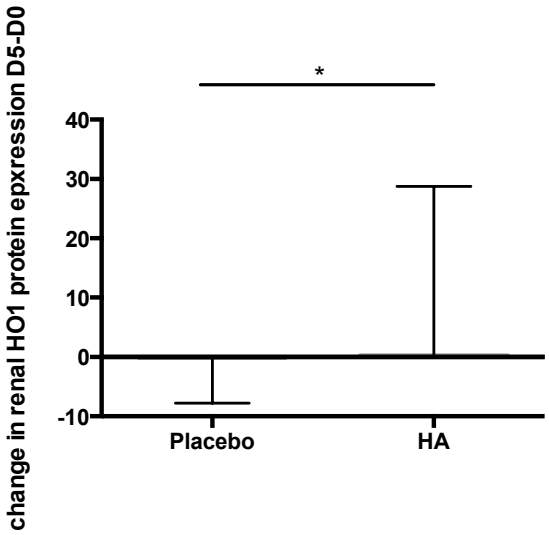
$$\% \text{ renal HO-1 protein} = \left(\frac{\text{renal HO-1 protein}}{\text{total renal protein}} \right) \text{x100}$$

Then the difference between paired samples was calculated and compared between groups:

Difference = % renal protein D5 - % renal protein D0

When HO-1 protein upregulation was expressed as the difference in % HO-1 expression on D5 compared with D0, HA upregulated protein by 0.21% (0.1- 0.6) compared to placebo of -0.03% (-1.3- 0.1) (figure 3- 15 a & b). This was a statistically significant difference supporting HA as an upregulator of HO-1 protein in renal tissue although it must be highlighted that actual values are small (p= 0.017).

a)



b)

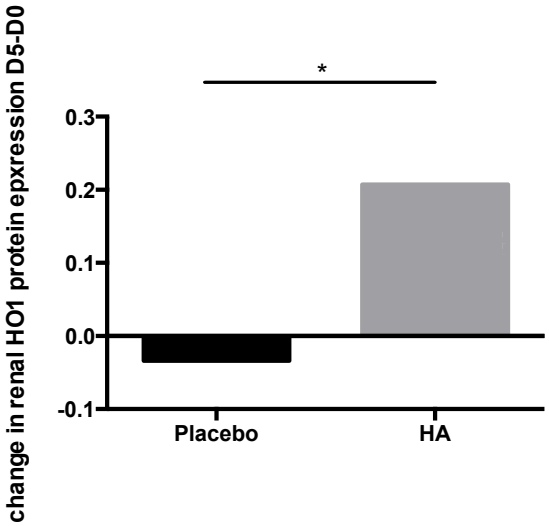


Figure 3- 15 Difference in % HO-1 renal protein between D5 and D0 a) with, b) without IQR

In addition, a comparison of proportions test was used to compare the numbers who upregulated HO-1 protein in each group (table 3- 4). 30% placebo group (95% CI; 1.6- 58.4) upregulated HO-1 protein vs. 75% HA group (95% CI; 50.5- 99.5). The 45% difference (95% CI; 7.5- 82.5) was significant (p=0.08).

Table 3- 4 Comparison of proportions of renal tissue HO-1 protein upregulation

	Treatment			
	Placebo (n=10)		HA (n=12)	
Up-regulation HO-1 protein	**N**	**%**	**N**	**%**
No	7	70	3	25
Yes	3	30	9	75

3.4.2 *HO-1 mRNA expression*

The renal tissue results were analysed in the same way as the PBMC mRNA calculations using $2^{-\Delta\Delta C_T}$ method to determine the fold increase of day 5 tissue compared to baseline (back table tissue). There was a 2.02 (0.20- 4.03) fold increase in the HA group compared to 1.68 (0.75- 10.39) fold in the placebo group. This was not significant (p= 0.451) (figure 3- 16).

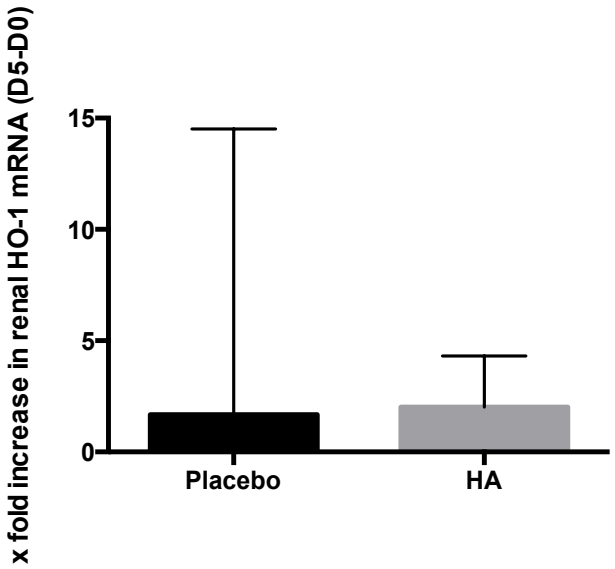


Figure 3- 16 D5 HO-1 mRNA upregulation expressed as fold increase from D0

In contrast with the protein results, when the comparison of proportions test was performed; 53.8% of HA group upregulated HO-1 in renal tissue vs. 60% of placebo group. The Fishers test was performed on the difference of 6.2% (CI: -34.5 – 46.90) and this was also not significant (p= 0.994).

Table 3- 5 Comparison between renal tissue HO-1 mRNA upregulation

	Treatment			
	Placebo (n=10)		HA (n=13)	
Up-regulation HO-1	**n**	**%**	**N**	**%**
No	4	40	6	46
Yes	6	60	7	53.8

3.4.3 *Histology*

An independent blinded Consultant Pathologist scored renal tissue samples as per previously defined method (section 2.3.2.6).

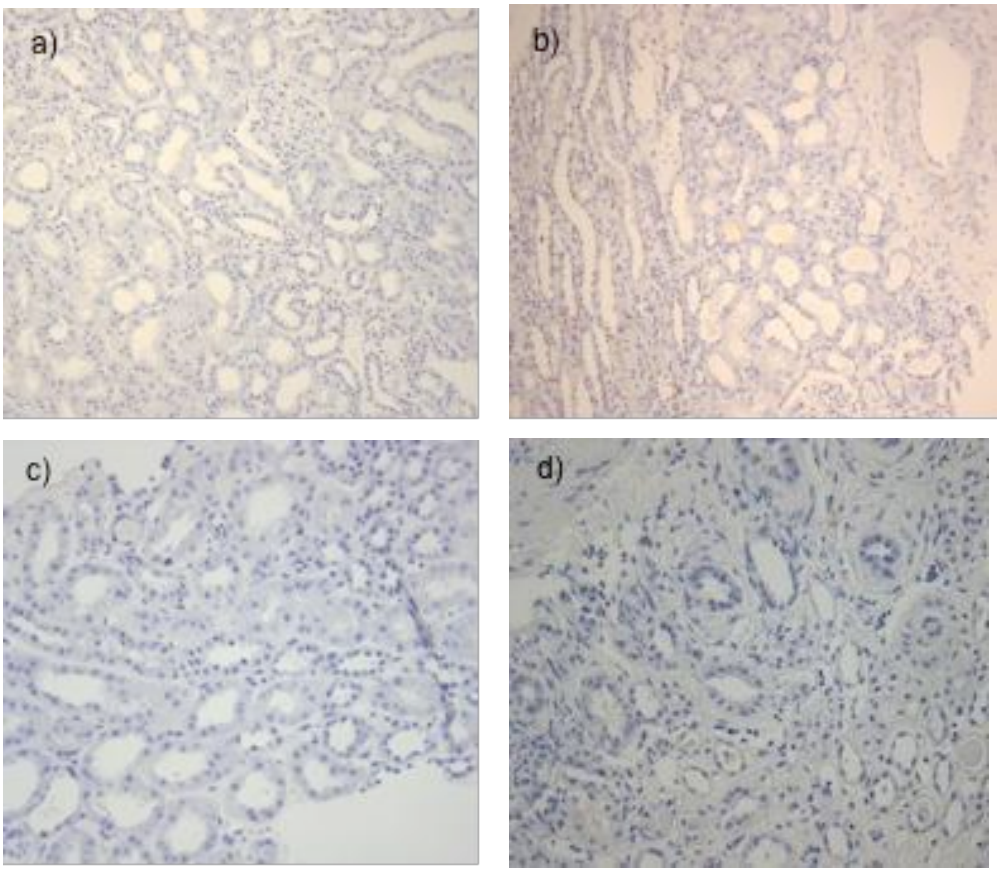


Figure 3- 17 Comparison images of healthy graft kidney at a) x10 magnification, c) x20 and injured tissue at b) x10 magnification, d) x20

There were 21 paired samples for analysis (8 in placebo and 13 in HA group). The D0 renal tissue score was compared with D5 score and cross tabulations were created for the available samples divided by group.

Table 3- 6 Cross tabulation of D0 (back-table) and D5 (day 5) biopsy sample pathology scores for a) placebo b) HA (inad = inadequate, mod = moderate)

a) Placebo

Placebo	**D0**											
	Inad sample		0 (no injury)		1 (mild)		2 (minor)		3 (mod.)		4 (severe)	
	N	**%**	**N**	%	**N**	%	**N**	**%**	**N**	**%**	**N**	**%**
Number with transplant	**3**	**100**	**0**	**-**	**6**	**100**	**6**	**100**	**3**	**100**	**0**	**-**
D5												
Patient unwell	-	-	-	-	1	17	1	17	-	-	-	-
Patient refused	2	67	-	-	1	17	1	17	1	33	-	-
Inad sample	-	-	-	-	1	17	-	-	1	33	-	-
0 (no injury)	-	-	-	-	-	-	-	-	-	-	-	-
1 (mild)	1	33	-	-	2	33	-	-	1	33	-	-
2 (minor)	-	-	-	-	1	17	-	-	-	-	-	-
3 (mod)	-	-	-	-	-	-	2	33	-	-	-	-
4 (severe)	-	-	-	-	-	-	2	33	-	-	-	-

b) HA

HA	D0											
	Inad sample		0 (no injury)		1 (mild)		2 (minor)		3 (mod.)		4 (severe)	
	N	**%**	**N**	%	**N**	%	**N**	**%**	**N**	**%**	**N**	**%**
Number with transplant	**2**	**100**	**3**	**100**	**2**	**100**	**3**	**100**	**7**	**100**	**2**	**100**
D5												
Patient unwell	1	50	-	-	-	-	-	-	-	-	1	50
Patient refused	-	-	-	-	-	-	-	-	2	29	1	50
Inad sample	-	-	-	-	-	-	-	-	-	-	-	-
0 (no injury)	-	-	-	-	-	-	1	33	-	-	-	-
1 (mild)	1	50	1	33	-	-	-	-	2	29	-	-
2 (minor)	-	-	1	33	-	-	-	-	-	-	-	-
3 (mod)	-	-	1	33	-	-	1	33	3	43	-	-
4 (severe)	-	-	-	-	2	100	1	33	-	-	-	-

A further analysis compared D5- D0 scores between groups. A negative result means the score improved (D5 kidney was less injured than at D0) and a positive result represented deterioration in the health of the kidney at D5. Although you were more likely to see an improvement in health of the kidney after HA treatment, there was no significant difference between the treatments as assessed by chi^2 test (Fishers p= 0.63).

Table 3- 7 Difference in pathology scores by group. (No -1 score in this analysis.)

Score = D5-D0		**Treatment**			
		Placebo		**HA**	
		N	**%**	**N**	**%**
Total available for analysis		**8**	**100**	**13**	**100**
Difference in pathology score	**-2**	1	13	3	23
	0	2	25	3	23
	1	3	38	2	15
	2	2	25	2	15
	3	-	-	3	23

The raw D5 scores were also compared with no significant difference between the groups by chi^2 test (Fishers p= 0.95) (table 3- 8) or median scores (p= 0.85) (figure 3- 18).

Table 3- 8 D5 pathology scores by group

		Treatment			
		Placebo		HA	
		n	%	N	%
Number with D5 pathology score		**9**	**100**	**14**	**100**
Pathology score	**0**	-	-	1	7
	1	4	44	4	29
	2	1	11	1	7
	3	2	22	5	36
	4	2	22	3	21

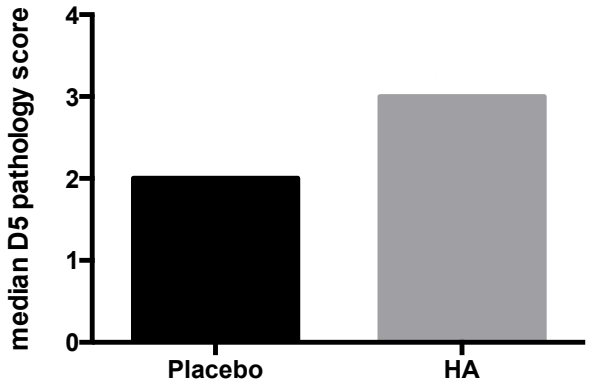


Figure 3- 18 Comparison of D5 pathology score between placebo and HA

3.5 Urine biomarkers

For both NGAL and KIM-1, median raw values for each day were compared between groups. A non-parametric Mann-Whitney test was performed and there was no significant difference in median biomarkers between groups for either biomarker at all days. This was true for both raw values and urinary biomarker: creatinine ratio (UBCR) data. Only raw value graphs are shown (figure 3- 19 & 3- 20).

Table 3- 9 Results of urinary biomarker analysis

	Max value (placebo)	**Max value (HA)**	**P-value**
KIM-1 (raw data)	8.8 $\pm$ 8.7	4.8 $\pm$ 3.9	0.17
NGAL (raw data)	638.5 $\pm$ 371.2	494.4 $\pm$ 375.1	0.15
KIM-1 (UBCR)	2.9 $\pm$ 4.6	2.6 $\pm$ 5.7	0.53
NGAL (UBCR)	266.7 $\pm$ 371.0	266.5 $\pm$ 295.6	0.60

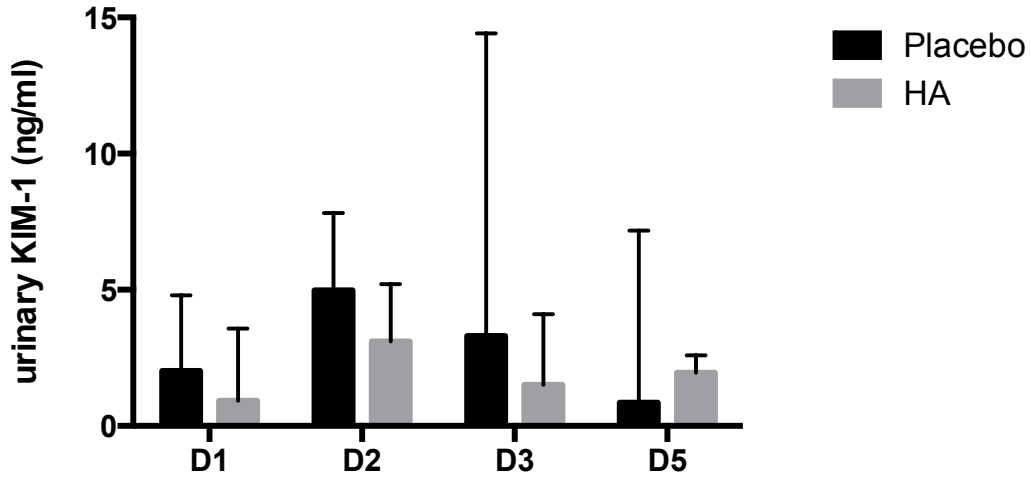


Figure 3- 19 Change in median raw urinary KIM-1 over 5 days with HA and placebo

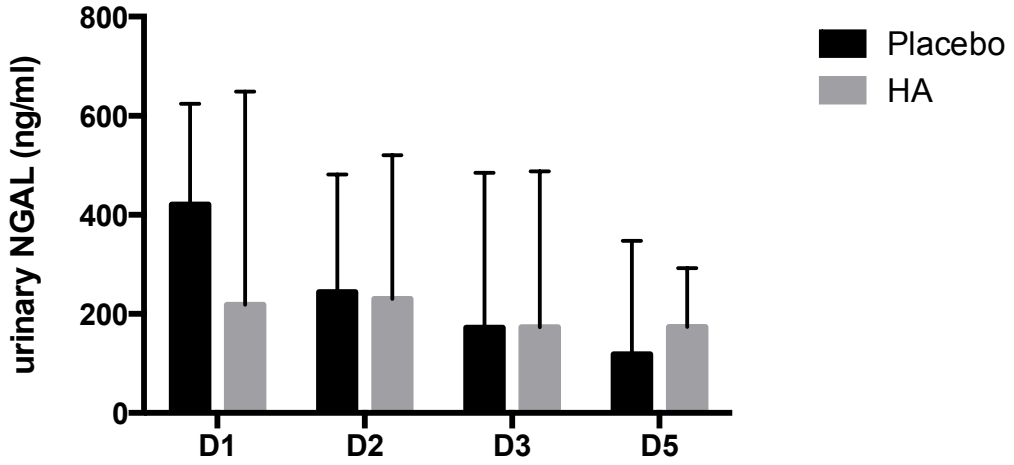


Figure 3- 20 Change in median raw urinary NGAL over 5 days with HA and placebo

Area under the curve analysis was also performed on the maximum raw and UBCR biomarker data to assess the usefulness of urinary biomarkers for detecting differences between the groups (figure 2- 21). In AUC analysis, the closer the value is to 1, the better it is at distinguishing between groups. A value of 0.5 is expected by chance.

The KIM-1 raw analysis gave an AUC value of 0.647 (95% CI; 0.447 - 0.847).
The KIM-1 UBCR analysis gave an AUC value of 0.569 (95% CI; 0.364 - 0.773).

The NGAL raw analysis gave an AUC value of 0.655 (95% CI; 0.460 - 0.850).
The NGAL UBCR analysis gave an AUC value of 0.443 (95% CI; 0.233 - 0.653).

Therefore, there was no evidence that KIM-1 or NGAL were accurate or useful at distinguishing between the treatment groups.

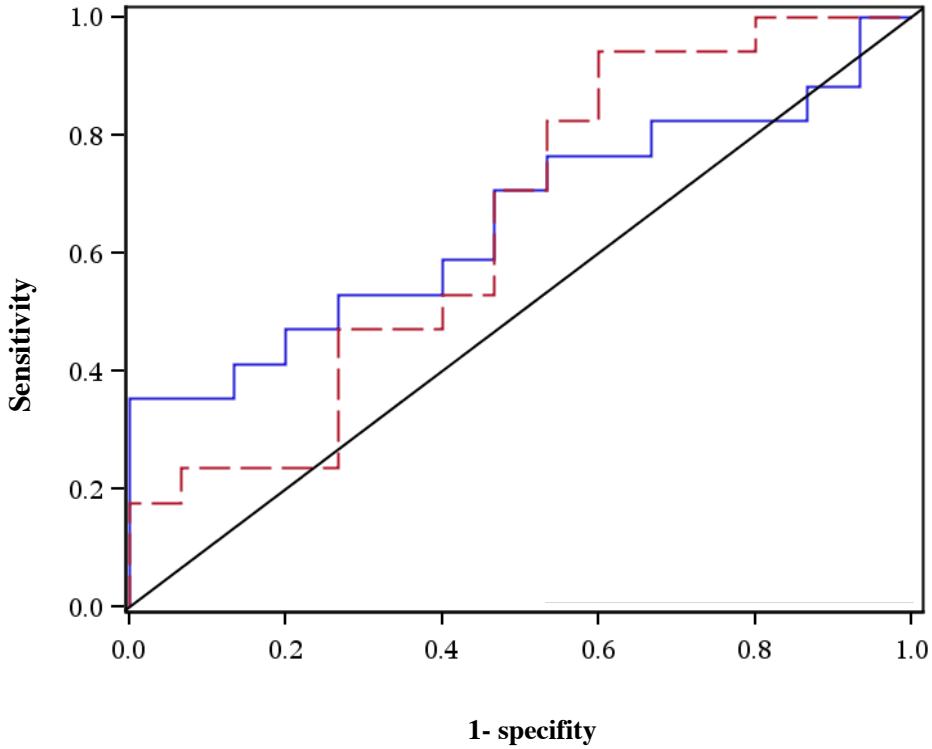


Figure 3- 21 AUC analysis to investigate the value of raw KIM-1 and NGAL for predicting treatment groups **NGAL (max) (0.6549)** **KIM-1 (max) (0.6471)**

In another analysis, participants were subdivided into 2 groups regardless of treatment: those with DGF and those without. There was no evidence that either raw or UBCR maximum KIM-1 or NGAL values were accurate or useful at predicting DGF (regardless of treatment group) (figure 3- 22).

The AUC values were 0.683 (95% CI; 0.472- 0.893) for maximum NGAL and 0.583 (95% CI; 0.374 – 0.793) for maximum KIM-1.

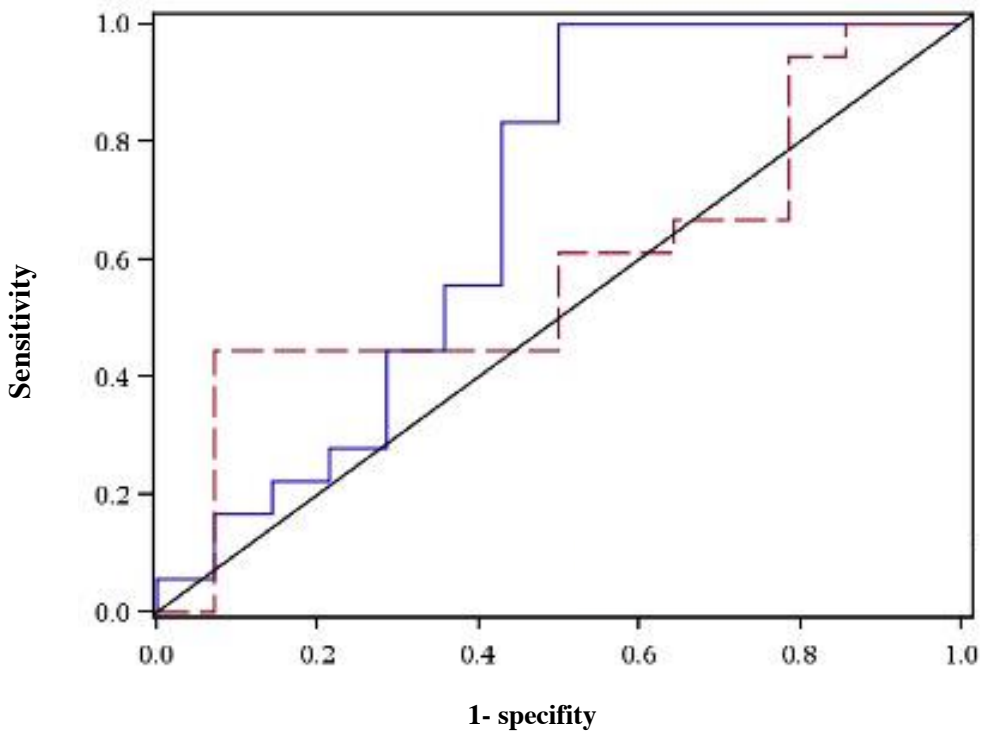


Figure 3- 22 AUC analysis to investigate the value of raw KIM-1 and NGAL for predicting DGF **NGAL (max) (0.6825)** **KIM-1 (max) (0.5833)**

3.6 Graft function

3.6.1 Delayed graft function (day 7)

The percentage of participants with delayed graft function (DGF) was compared between the groups. DGF was defined as "as an increased or stable serum creatinine, or a decrease of less than 10% per day in three consecutive days in the first week after transplantation". 12 participants had DGF in the placebo group and 10 in the HA group. This equated to 66.7% in placebo and 52.6% in HA. The 14% difference (95% CI; -17.2 – 45.3) was not statistically significant (p=0.38).

There were the same numbers of participants who required dialysis in each group (4), which equalled 22.2% in placebo group and 21.1% in HA group.

The proportion of participants who had a functioning renal transplant at day 7 was also calculated. The day of function was defined using the criteria "dialysis-free and >10% decrease in creatinine compared to previous day". There was no statistical difference between the groups: 55.6% in placebo and 57.9% in HA group, difference -2.3% (95% CI; -34.3 – 29.6) (p=0.89).

3.6.2 *30 day and 90 day follow-up*

Table 3- 10 Follow-up results

30-day follow-up	**Placebo (n=17)**	**HA (n=19)**
Number of participants dependent on dialysis	2 (11)	1 (5)
Evidence of rejection at day 30	1(6)	2 (11)
90-day follow-up		
Number without primary function	2 (11)	1 (5)
Evidence of rejection at day 90	1(6)	2 (11)

There was no significant difference in either mean or median creatinine values between the groups on day 30 or day 90.

At day 30, median creatinine (figure 3- 23) in the placebo group was 120 (103-197) and 138 in the HA group (118- 207). At day 90, median creatinine was 116 (90- 151) in the placebo group and 147 (103- 171) in the HA group.

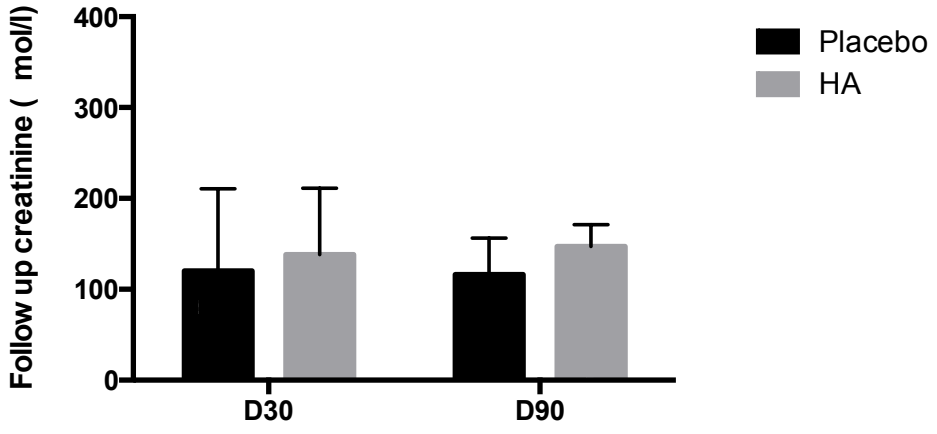


Figure 3- 23 Comparison of median creatinine from day 30 and day 90

On day 30 and on day 90, the difference in mean creatinine between treatment groups was also not significant: day 30 was -23.4: placebo (178.6) and HA (202.0), (p= 0.67) and at day 90 was -46.3: placebo (129.4) and HA (175.7) (p=0.21).

Chapter 4.

Discussion

4.1 HOT Study conclusion

Summary of key findings

The principle aim of the HOT (Heme Oxygenase-1 in renal Transplantation) study was to determine if pre-treatment of recipients of deceased donor renal transplants with heme arginate (HA) was feasible and safe. The primary outcome was to investigate if HA could upregulate heme-oxygenase 1 (HO-1) in recipient macrophages and this was achieved safely and effectively. The secondary outcomes explored the effect of HA on renal tissue structure and HO-1 expression, urinary biomarkers and renal function.

There was no evidence that HA was beneficial to renal structure or function. There was a trend to reduced excretion of KIM-1 and NGAL after HA treatment but this did not reach significance. Although the trend for reduced DGF rates was not statistically significant, the study was not powered to this effect. Given the prominence of HO-1 in many different IRI pathologies, the study generates several important results. Larger studies are required to investigate if upregulated HO-1 can translate to improved functional outcomes. There are very few novel drugs that make it to CTIMP trials in renal transplantation and HA presents another option that warrants further investigation.

4.1.1 HOT study overview

4.1.1.1 Recruitment

The study recruited the 40 participants in the pre-stated time frame. It was anticipated that the study would recruit about 60% of all admissions for renal transplant and out of 76 admissions we recruited 53% (figure 3- 1). There were more ineligible participants than expected, reflecting the increasing complexity of transplant workload (table 3-1), but an increased departmental workload meant that the study met its target (figure 3- 4).

The study had 100% follow-up, which, while impressive, reflects its intensive and short-term nature. Two participants declined the second infusion and 14 declined biopsies or blood samples but because these participants still gave consent to collect clinical data, they remained in the study and so there were no withdrawals (figure 3- 2 and 3- 3).

The study population was representative of the renal transplant population and therefore, the results are applicable to other renal transplant units. The study groups were comparable and there was no significant difference in identified characteristics (table 3- 2).

4.1.1.2 Safety of HA

HA is a ferric form of heme, which is licensed to treat acute porphyria. The HOT study was interested in another function: HO-1 induction. Compared to other available hemin preparations, HA is reported to be less thrombotic and vasculotoxic (Balla, Balla et al. 2000). This is mainly due to the fact that in HA, hemin is liganded to arginine and, as a result, the potentially harmful effects of ferric heme are reduced (Tenhunen, Tokola et al. 1987).

This was supported by the HOT study because there were no adverse reactions to or unexpected adverse events after HA (table 3- 3). In this study, the adverse event profile was similar between groups and HA was well tolerated. This expands current knowledge as, prior to this, HA had only been formally tested in healthy male volunteers and there was no recorded evidence of use in patients with end stage renal disease (ESRD) and immunosuppression.

The HOT study dose regime (3mg kg^{-1}) was chosen because it was within the safe licensed dose and is known to be sufficient to induce PBMC HO-1 *in vivo* and *in vitro*. Healthy volunteer studies found that PBMC HO-1 protein expression peaked at 24 hours after HA exposure and was declining at 48 hours. Therefore, the second dose was given to maximise exposure to the renal graft (Doberer, Haschemi et al. 2010).

4.1.2 *Heme-oxygenase 1 upregulation*

As explored in the introduction, HO-1 is induced in response to multiple stimuli including hypoxia and ischaemic cytokines so it was anticipated that all participants would experience an upregulation in HO-1 after transplantation (Shimizu, Takahashi et al. 2000). Heme is a well-known HO-1 inducer. Free heme binds to hemopexin and the complex then undergoes endocytosis where the intracellular heme can activate HO-1 transcription through Bach1 and Nrf2. The study, therefore, scrutinized whether the amount of HO-1 upregulation differed between groups.

4.1.2.1 PBMC population

In the HOT Study, immunohistology, ELISA and qRT-PCR confirmed PBMC HO-1 upregulation.

HA treatment increased PBMC HO-1 protein and mRNA significantly more than placebo treatment and restates results from the phase I studies (Bharucha, Kulkarni et al. 2010, Doberer, Haschemi et al. 2010).

The primary outcome established that HA treatment upregulated HO-1 protein 10 times greater than placebo at 24 hours ($p = <0.0001$) (figure 3- 5). This exceeded the findings of the healthy volunteer study that reported HO-1 protein expression five times baseline at the same time point (Doberer, Haschemi et al. 2010). This may be explained by the increased ischaemic insult suffered by our patients compared to healthy individuals in a controlled setting. The pattern of HO-1 PBMC protein expression was also as expected; there were two discrete peaks after the HA infusions with a return to near baseline expression after 24 hours (figures 3- 6, 3- 7).

Previous research found PBMC HO-1 mRNA was elevated in acute inflammatory illnesses, which was given as evidence of HO-1 as a mechanism for limiting excessive cell or tissue injury in the presence of oxidative stress (Yachie, Toma et al. 2003). The HOT study supported that conclusion because even the placebo group had a 1-fold increase in HO-1 mRNA at 24 hours post-infusion reflecting the oxidative stress of surgery (figure 3- 8). HA had an additive effect and resulted in a significant 2-fold upregulation of HO-1 mRNA ($p=0.02$). These results echo the findings from the previous healthy volunteer study, which reported a 2-fold increase in PBMC HO-1 mRNA 24 hours after HA, which had reduced to 1.5-fold increase at 48 hours. In the HOT study, the pattern of mRNA upregulation was not as predicted because although there was a clear peak at 24 hours, this was not significant after the second infusion (figure 3- 9 and 3- 10). This anomaly may be due to the $2^{-\Delta\Delta C_T}$ method used because all samples were referenced to baseline (day 0). It may be more appropriate to normalise day 3 results to day 2 to elicit any evidence of upregulation.

There have been many methods described to detect differences in HO-1 expression or upregulation including direct measures such as leucocyte protein concentration and indirect surrogate measures, for example plasma bilirubin, expired carbon monoxide (CO), nasal swab mRNA or urinary HO-1. Bharucha et al found increased HO-1 protein in plasma and increased HO-1 activity in healthy volunteers after HA infusion but HO-1 upregulation in PBMCs was considered a better measure of the cytoprotective action and the HOT study adopted this method as suggested by Doberer (Doberer, Haschemi et al. 2010).

The urinary method used to evaluate bioflavinoids as potential HO-1 upregulators has not been validated or replicated and would not have been suitable for renal transplant recipients (Shoskes, Lapierre et al. 2005). In the healthy volunteer study that investigated sulforaphane as a potential HO-1 upregulator, the amount of HO-1 mRNA in nasal aspirate was used as a primary endpoint. However, although this non-invasive technique is attractive, the specificity and sensitivity is unknown and the relationship between nasal mRNA and cellular physiology is unclear (Riedl, Saxon et al. 2009).

4.1.2.2 Renal samples

4.1.2.2.1 *Renal macrophages*

The HOT study was the first clinical study to examine the transplanted kidney for HO-1 upregulation by qRT-PCR and immunohistochemistry (IHC) (figure 3- 11).

There were similar numbers of macrophages in back table tissue regardless of treatment group (p=0.956) (figure 3- 12). As expected after an ischaemic insult, there was an increase in the number of macrophages in the day 5 tissue compared to back table. The increase is due an influx of recipient macrophages infiltrating the graft. Human data is lacking but this has been shown in animal models of renal IRI (Jo, Sung et al. 2006). When the role of macrophages in renal transplant studies was reviewed in Vascular Cell the authors described the infiltration of the graft by phenotypically distinct macrophages in different IRI phases (Jiang, Tian et al. 2014).

At day 5, there were more interstitial macrophages detected in the HA treatment group although this was not significant (p=0.13) (figure 3- 12).

There were no HO-1 positive CD68 cells in the back table biopsy samples and therefore the significant population detected in all the day 5 samples were HO-1 expressing, recipient macrophages infiltrating the graft. In agreement with previous animal work, there were significantly more HO-1 positive CD68 cells in the HA treated group (p=0.012) (figure 3- 13).

4.1.2.2.2 *Renal tissue*

The HOT study demonstrated that HA treatment significantly increased the concentration of HO-1 protein in renal tissue as measured by IHC (p=0.017) (figure 3- 14 and 3- 15). However, the differences between the groups were small and closely clustered around 0 with the majority of samples having no difference in HO-1 expression between back table and day 5 (table 3- 4). There was no evidence that HA resulted in an upregulation of HO-1 mRNA in renal tissue either as comparing the median difference (p=0.45) (figure 3- 16) or comparison of proportions test (p= 0.994) (table 3- 5).

These findings can be explained. Renal tissue sampling was problematic. The initial plan was to analyse HO-1 protein in renal tissue by Western Blot but the samples were too small for accurate and reproducible protein analysis.

There are a number of possible explanations why the renal tissue results were not as convincing as expected:

1. There was a real, significant upregulation of renal HO-1 after HA but it was not captured by the HOT study analysis because the number of paired renal samples was too small (only 22 out of 37 potential samples). Eight participants with primary function declined the day 5 biopsy. An additional four participants were not considered well enough or had contraindications for renal biopsy. This relatively low take-up was not unexpected because a renal biopsy has recognised risks. The responses will help to plan the follow-on study, which may not include a day 5 biopsy.

2. There was a real, significant upregulation of HO-1 after HA but it was not seen because the sample quality was too poor. Despite expert examination prior to freezing, the subtype of renal tissue was not standard or guaranteed. HO-1 is preferentially upregulated in tubular cells, specifically the outer stripe of the medulla (OSOM), but the amount of this tissue varied in each sample.
   The tissue samples were also small and this impacted on preservation and analysis quality. Although there was ethical approval for two renal biopsies at back table and two on day 5, the experience and preferences of the surgeons and radiologists taking the biopsies affected the sample size and quality. The qRT-PCR analysis of the renal tissue occasionally generated C_T values at the extent of the machine capability, which may have had a negative influence on reliability.

3. HO-1 mRNA upregulation occurred but the timing of the protocol biopsy missed it. mRNA is a more transient marker than protein and the half-life of HO-1 mRNA in *in vitro* renal cells treated by hemin is only around four hours (Hill-Kapturczak, Sikorski et al. 2003). Animal models show that, following heme stimulation, renal HO-1 mRNA expression is first measurable at six hours and has returned to baseline by 24 hours (Shimizu, Takahashi et al. 2000). HA also only has a half-life of ten hours. The HOT study attempted to extend the effect of HA by repeating the dose on day 2 but this was untested and may still be too low.

   The timing of the biopsy also varied between subjects because the biopsy service was not available at the weekend. This led to 7/25 biopsies being performed on day 6-post infusion and this may have negatively impacted on the results.

In addition it should be highlighted that the renal tissue protein and mRNA results may be of limited value because the predominant source of HO-1 positivity in human renal tissue comes from infiltrating macrophages (Gueler, Park et al. 2007). It was confirmed in **4.2.2.2.1** that HA had a positive effect on renal macrophage HO-1 expression.

4.1.3 *HA effect on histological tissue*

In contrast to experimental models, there was no evidence that HA had a positive effect on the histological health of the kidney at day 5 (p=0.95) (Ferenbach, Nkejabega et al. 2011)(table 3- 8 and figures 3- 17 and 3- 18). The first two reasons quoted in **4.1.2.2.2** are also valid to explain this finding.

Crucially the clinical study results do not replicate animal models because human transplant is more complex involving donor and retrieval factors, immunosuppression and additional disease processes. The HOT study samples were also potentially more complicated than those analysed in preclinical studies because the later time point also captures features of resolution and repair in addition to the original injury.

The differences in injury scores between paired samples (back table biopsy and day 5 biopsy) were also compared (tables 3- 6, 3- 7 and 3- 8), and although HA was more likely than the placebo group to result in an improvement in injury scores, the sample was too small for formal analysis (p=0.63). A larger sample size is required to investigate whether this finding is genuine or due to small numbers.

4.1.4 *HA effect on urinary biomarkers*

Serum creatinine is an unreliable indicator of acute kidney injury and does not reflect the degree of damage until it plateaus, which may be several days post-operatively (Parikh, Jani et al. 2006). Creatinine kinetics varies between individuals and may be influenced by multiple non-renal factors, which confuses the interpretation of renal function. Clinicians are still looking for a better assay that would be prognostic for DGF, allowing earlier and targeted management (Hall, Yarlagadda et al. 2010). A biomarker is a biologic characteristic that is measured and evaluated objectively as an indicator of normal biologic processes, pathological processes or responses to an intervention (Coca and Parikh 2008). If proven, analysis of selective biomarkers could alter patient management and serve as outcomes for transplant drug development (Hall, Doshi et al. 2012).

The HOT study explored whether HA had an impact on two urinary biomarkers: neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury marker-1 (KIM-1). These biomarkers were chosen because they have been shown to be applicable in transplantation and have different pathways of production to capture different mechanisms of injury. Commercial, validated assays are also available.

There were no statistical significant differences in maximum or median NGAL or KIM-1 between groups, both using the raw data and when allowing for differences in urine concentration (figure 3- 19 and 3- 20). The maximum NGAL and KIM-1 levels were lower in HA group indicating reduced renal injury but not significantly so (table 3- 9).

The analysis of biomarkers was limited in some patients by negligible urine production. For many patients with DGF, there was no urine available to test and this therefore may have skewed results. As mentioned previously, urinary biomarker results were not taken into account when calculating the study size and therefore it may be underpowered to see a difference in these samples. AUC analysis was also not able to distinguish between participants with DGF in this small sample (figure 3-21).

Urinary biomarkers, including NGAL and KIM-1, are proteins that are released from damaged kidney cells into the urine and therefore, increase after injury. They peak earlier than creatinine and have been considered in many different renal injury scenarios as potentially more timely, accurate and less invasive predictors of injury (Hall, Doshi et al. 2012). However, a consensus has yet to be reached on the usefulness of urinary biomarkers and research continues. They are not currently part of routine UK clinical practice.

NGAL is one of the most promising markers. It is expressed at low levels in normal kidneys but is upregulated in damaged tubules, from which it escapes into the urine (Mishra, Dent et al. 2005). In addition to its role as a marker of disease, NGAL is also recognised as an iron-transporting protein and has been investigated as therapy for acute renal injury in mice (Mishra, Mori et al. 2004). It may also be involved in HO-1 upregulation because, while NGAL offers protection in acute kidney injury, this protective effect is lost when HO-1 is inhibited (Mori, Lee et al. 2005).

One group found that they were able to accurately predict the need for dialysis within the first week and also the 1-year graft function from the level of NGAL in a recipient's urine collected on the first day post-transplant using an adult study population four times larger than the HOT study (Hall, Yarlagadda et al. 2010, Hall, Doshi et al. 2012). Another group found similar results in Finnish deceased donor recipients in the first few weeks supporting the hypothesis although this was not supported in longer-term follow-up (Hollmen, Kyllonen et al. 2011). Other studies were limited by small sample sizes of mixed deceased and living donors (Mishra, Ma et al. 2006, Parikh, Jani et al. 2006). The recent review by Ronco advocates the use of routine NGAL testing in a variety of clinical settings including early renal transplant but larger studies are required to corroborate sensitivity and specificity (Ronco, Legrand et al. 2014).

KIM-1 is a transmembrane protein, which is undetectable in normal kidneys and overexpressed in proximal tubular cells during ischaemic and nephrotoxic acute kidney injury making it an ideal biomarker (Coca and Parikh 2008, Bonventre and Yang 2010). The level of KIM-1 correlates with kidney function post-transplant (Malyszko, Koc-Zorawska et al. 2010). One group found that urinary KIM-1 was an independent predictor of graft loss in renal transplantation although this study took place in an outpatient setting and therefore could not comment on the value of KIM-1 at detecting DGF in the first week post-transplant (van Timmeren, Vaidya et al. 2007). There is also evidence that urinary KIM-1 levels from recipients under investigation for acute rejection correlate to declining renal graft function although the same was not true of NGAL (Szeto, Kwan et al. 2010).

As evidence that further clarification is needed, the Hall study that supported NGAL found that KIM-1 was not of value at predicting renal recovery (Hall, Yarlagadda et al. 2010).

The HOT study was too small to make assumptions about the value of these two urinary biomarkers after transplant and a global conclusion has yet to be reached about the use of biomarkers as predictors of DGF. The HOT study chose two well recognised biomarkers but there are many more under investigation such as IL-8 (a proinflammatory cytokine) and clusterin (a protein associated with clearance after apoptosis) (Pianta, Peake et al. 2014). Each biomarker may offer different predictive insights and, as more biomarkers are identified, tested and validated, it may become apparent that the best solution is a panel of biomarkers that could stratify future function and aid clinician decision-making. If biomarkers are shown to provide specific and sensitive prognostic information, they could guide treatment planning and improve patient information for better clinical care. NGAL and KIM-1 are of particular interest as bedside testing devices are now available (Field, Dronavalli et al. 2014).

4.1.5 *HA effect on function*

4.1.5.1 Delayed graft function (DGF)

Delayed graft function is clinically important and there is evidence that while one episode of dialysis post-transplant has no effect on graft survival, patients requiring more than one episode of dialysis have inferior clinical outcomes (Yarlagadda, Coca et al. 2009, Jayaram, Kommareddi et al. 2012). DGF occurs in between 20 to 35% of recipients and the mechanisms are not fully understood (Siedlecki, Irish et al. 2011). With improved strategies to prevent acute rejection, DGF is the most important factor impacting on long-term allograft survival (Serur, Saal et al. 2011). In the HOT study, there were fewer cases of delayed graft function after HA treatment but this was not statistically significant (p=0.38). This may be because the study was not designed to compare functional outcomes and the numbers analysed were not large enough to detect the small difference in function. However, the DGF analysis was performed due to its relevance for clinical implications.

A variety of clinical definitions exist for DGF; Yarlagadda identified 18 (Yarlagadda, Coca et al. 2008). A clinical trial is currently recruiting in Korea to compare various definitions of DGF and to find the definition that best predicts graft function and survival (NCT02080117 clinicaltrials.gov).

In the HOT study, DGF was defined as an increased or stable serum creatinine, or a decrease of less than 10% per day in three consecutive days in the first week after transplantation: the “functional” definition as advocated by Moore (Moore, Shabir et al. 2010).

It has been acknowledged that the dialysis-based definition is subjective and may represent clinician choice rather than true graft function (Sharif and Borrows 2013). Moore et al compared these two DGF methods and demonstrated that functional DGF but not dialysis requirement was associated independently with subsequent death-censored transplant failure. The functional method is also easier to accomplish and validate for large or retrospective studies, as case note examination is not required. For completeness, the need for dialysis was also compared and it was the same between groups.

It is recognised that regardless of definition, DGF is difficult to predict and trials attempting to foretell DGF as an outcome measure have had limited success as reviewed by Sharif (Sharif and Borrows 2013). Dopamine remains one of the only agents to have proven success in reducing DGF when given to donors (Schnuelle, Gottmann et al. 2009). Neither recipient treatment with erythropoietin (Martinez, Kamar et al. 2010, Sureshkumar, Hussain et al. 2012) nor pre-treatment of deceased donors with steroids (Kainz, Wilflingseder et al. 2010) or N-acetylcysteine (Orban, Quintard et al. 2014) saw a difference in DGF despite evidence that the drugs protected kidneys in preclinical studies.

4.1.5.2 Day 30 and day 90 follow-up

There was no difference in function at follow-up and in fact the placebo group had a lower mean and median creatinine but there was no statistically significant difference (figure 3- 23). The numbers of participants with rejection were too small to compare but there was no evidence that HA increased the risk of rejection (table 3- 10).

4.1.6 *Assessment of genotype impact in HOT study*

The GT polymorphism that modulates HO-1 gene transcription varies in size and allows individuals to be defined as short allele "S" (<25/ 27/ 28 repeats) or long allele "L"(> 25/ 27/ 28) responders. The longer GT repeat corresponds to reduced transcription activity and increased risk for a variety of diseases (Chen, Lin et al. 2002, Exner, Minar et al. 2004). Initial studies showed that S/S or S/L grafts had better outcomes after renal transplantation (Exner, Bohmig et al. 2004). However, as research interest increased, the significance of HO-1 genotype appeared to decrease and Exner et al's initial results have not been replicated. Separate studies from Belfast (Courtney, McNamee et al. 2007), Finland (Turpeinen, Kyllonen et al. 2007) and Greece (Katana, Skoura et al. 2010) found that neither donor nor recipient genotype significantly influenced graft or recipient survival. A retrospective study looking at a variety of inflammation genotypes including HO-1 could not find a link between HO-1 genotype and DGF rates after deceased donor kidney transplantation (Israni, Li et al. 2008).

Doberer et al examined the impact of genotype in their healthy volunteer study and found that GTn length polymorphism had no effect on the extent of HO-1 induction in PBMCs. In fact, they found the opposite, with HO-1 mRNA greater in those homozygous for long allele (L/L) (Doberer, Haschemi et al. 2010). In the HOT study, all participants were assigned to one of three genotype groups: S/S, L/L, S/L and the distribution corresponded with previously quoted prevalence patterns (Doberer, Haschemi et al. 2010). There were no participants with S/S genotype in the HA group so further subgroup analysis was not possible (table 3-2).

For interest, S/L vs. L/L analysis was performed and the difference in PBMC HO-1 protein upregulation was not significant ($p=0.59$).

Doberer suggested that different PBMC subtypes may respond to HA differently and that further PBMC subpopulation analysis may be required before the genotype hypothesis is dismissed completely (Doberer, Haschemi et al. 2010). This conclusion could equally apply to the HOT study.

In a review of HO-1 genotype in renal injury, Courtney et al hypothesised that there are other gene variants that confer protection in renal transplantation, which may compensate for a genetic predisposition towards lower HO-1 production in L/L individuals. This would ensure that even if the HO-1 genotype did negatively alter the cellular response to injury, it would be masked clinically (Courtney and Maxwell 2008). This seems feasible given the strong importance of HO-1 as evident in the case of the HO-1 deficient individual and HO-1 -/- animals (Kawashima, Oda et al. 2002, Kovtunovych, Ghosh et al. 2014).

4.1.7 *Generalizability and limitations of HOT study*

Despite clear evidence that HA upregulated HO-1, it did not translate into structural or functional evidence of renal cytoprotection seen in many animal models and clinical studies (Katori, Busuttil et al. 2002, Tullius, Nieminen-Kelha et al. 2002, Blydt-Hansen, Katori et al. 2003, Lemos, Ijzermans et al. 2003, Wagner, Cadetg et al. 2003, Ollinger, Kogler et al. 2008, Ferenbach, Nkejabega et al. 2011).

The HOT study was a single-blind single-centre randomized trial but because of the blinding of surgeons, nephrologists and nurses in charge of the recipients and the blinding of data management and sample analysis, it seems unlikely that this would have introduced bias to the laboratory samples. Also given the clear definition of DGF in the HOT study, it is also unlikely that the treatment had any influence on clinical outcome. The study adhered to CONSORT guidelines for randomised control trials and therefore the results should provide high quality evidence.

The study population was representative and this should ensure that our conclusions can be safely and effectively translated to other renal transplant units.

There are a few possible explanations why HO-1 upregulation did not confer protection:

1. The sample size was too small to detect a difference in histological injury or rates of delayed graft function between the groups. The study was not powered to these endpoints so this was not unexpected and larger studies are planned.

There is also the challenge that the majority of cadaveric renal transplants have primary function, thus cannot get further clinical benefit from HA. This will increase the numbers required in any study aiming for a definitive clinical outcome.

2. It may be that two doses of HA were insufficient to give protection and potentially more HA is required to maximise and sustain the effect on macrophages and renal tissue. HO-1 expression increases with increasing HA doses (Doberer, Haschemi et al. 2010) and the license for porphyria permits four doses. The proposed alterations to the follow-up study are explored in **4.3.1**.

   The timing of the HA dose also varied and there was a wide variation in infusion-to-graft-reperfusion time between participants although the mean and median times were not statistically significant between groups. It is not known whether there is an optimal infusion time for maximal HO-1 effect in graft tissue. Further studies will hope to evaluate the benefits of a higher dose or longer exposure.

3. It is possible that the structural and functional results are genuine and HA has other effects in IRI that prevents the HO-1 upregulation having its anticipated effect. While the study showed an increase in the known anti-oxidative and vasodilatory agent HO-1, it did not measure biological surrogate markers of oxidative stress or haemodynamic parameters to confirm the HO-1 effect. Therefore, it may be that the upregulated HO-1 did not have its expected, proven and beneficial actions in this trial. This remains to be explored.

4. The HOT study was interested in HO-1 induction but there are reports that basal HO-1 activity is more important for antioxidant action and this has not been fully explored (Tsuchihashi, Livhits et al. 2006). The HOT study did not categorise participants into subgroups based on basal HO-1 expression but this may be appropriate for further studies. There was a wide range of baseline HO-1 expression (as seen in raw numbers in figure 3- 6) and this variation may be worth exploring.

5. It has also been proposed that the protective response of HO-1 may be limited and susceptible to being overwhelmed. It may be that once a level of injury has been reached, additional HO-1 induction cannot prevent further damage (Tsuchihashi, Livhits et al. 2006). Clinical renal transplantation delivers sizable, diverse insults to the kidney, which are not fully modelled in animal experimentation, and may explain why preclinical findings do not equate to human studies.

6. Further research may show that HO-1 upregulation does not have the same protective effect on all grafts. Pre-clinical studies showed that HO-1 upregulation by HA gives maximal protection in older mice but had less effect in younger animals (Ferenbach, Nkejabega et al. 2011). It may be that the "better" organs experience very little benefit from HO-1 upregulation because they are already optimal but the poorer "marginal" kidneys have either more to gain or more capacity for improve.

The HOT study did not have sufficient numbers of ECD organs (2 in placebo and 4 in HA) to analyse separately but larger studies, which should allow for subgroup analysis, will try to answer this question. If this is found to be the case then HA may be offered to a select group of recipients only and form part of a targeted treatment regime.

4.2 Future considerations

4.2.1 *Preconditioning as a method for preventing renal IRI*

The technique being tested in the HOT study is known as preconditioning, which can be either ischaemic (IPC) or pharmacological (Selzner, Boehnert et al. 2012). Preconditioning is defined as a situation when an animal is exposed to a stressor or stimulus at a non-toxic concentration in order to prepare it for a later encounter with similar larger stressors (heme in the case of the HOT study) (Bonventre 2002). Several models have shown that hemin pre-treatment is protective in acute injury including cerebral, cardiac and hepatic as reviewed by Lu (Lu, Chen-Roetling et al. 2014) but until now, this has not translated to human studies.

IPC was originally described in 1986 when it was found that a brief episode of non-lethal cardiac ischaemia protected canine cardiac muscle from ensuing prolonged cardiac ischaemia (Murry, Jennings et al. 1986). Cochrane et al also confirmed the phenomenon in renal injury when they found that the kidneys of rats that had received a small ischaemic stimulus prior to 45 minutes of ischaemia had improved function and less histological injury than the control group with no preconditioning (Cochrane, Williams et al. 1999). Research was furthered by the recognition that the IPC event need not occur directly to the organ of interest. Wever et al found that an ischaemic event to an animal's limb conferred the same preconditioning protection against IRI as a direct ischaemic event to the kidney (Wever, Masereeuw et al. 2013). This is known as remote ischaemic preconditioning (RIPC).

Despite the known beneficial effects of IPC and RIPC in many disease pathologies, clinical trial results have been mixed (Szwarc, Soullier et al. 2007, Huang, Shan et al. 2009). While initial results from clinical trials of IPC in liver transplant were positive (Amador, Grande et al. 2007) another group raised the issue of an "IPC paradox"; where IPC did more harm than good. (Koneru, Fisher et al. 2005, Koneru, Shareef et al. 2007). Although the initial randomised controlled trial (RCT) by Koneru et al in 2005 found no difference in recipient function or graft injury after a 5 minute period of liver ischaemia prior to liver donation in 62 donors, their follow-up study of 101 donors found that IPC prior to liver transplant actually increased IRI.

Three recent RCTs from Asia have investigated RIPC in renal transplant. Chen et al found no benefit in renal function at 72 hours after pre-transplant RIPC to either donor or recipient although accepted that study numbers were small (Chen, Zheng et al. 2013). In contrast, Kim et al detected an improvement in donor renal function at 24 hours after recipient RIPC treatment but this result was not sustained long term (Kim, Lee et al. 2014). However both of these trials were in living donors and it was acknowledged that the majority of the grafts were expected to have primary function. It was therefore suggested that either the sample size was increased or the studies were repeated in deceased donors at higher risk of IRI. This hypothesis was supported by a recent trial who found that when recipients of deceased donor renal grafts received an ischaemic stimulus, in the form of a brief external iliac artery occlusion at time of implantation, there was an improvement in creatinine and eGFR during the first two weeks post transplant. The improvement was not sustained beyond this but suggests potential (Wu, Feng et al. 2014).

Potential reasons behind the reported IPC paradox were explored in a review article by McCafferty et al who highlighted the problems of extrapolating the results from animal models to human studies (McCafferty, Forbes et al. 2014). They highlighted the differences in the study environments comparing the use of juvenile, male, inbred animals housed in controlled conditions with patients with multiple co-morbidities and medications recruited to clinical studies. Diabetes, hypercholesterolaemia, hypertension and obesity have all been shown to have an impact on preconditioning strategies and some medications act as pharmacological preconditioning agents, which further muddies the data. The best human conditioning protocol is also unknown and larger clinical trials are required to examine this potential area of interest. There is a multi-centre RCT led by University College London, which aims to recruit 400 living-donor transplant patients to look at the impact of RIPC on renal function post-transplant and the results are eagerly awaited (protocol ISRCTN30083294 RIPC in renal transplantation).

The results from the HOT study support HA as an alternative to other renal transplant pharmacological conditioning strategies that have not fulfilled their potential when translated to clinical studies. Despite giving protection in animal models, erythropoietin has not been shown to be cytoprotective in humans. A study comparing high-dose recombinant human erythropoietin to placebo found that, while adverse events were similar, there was no evidence of functional or histological improvements at short (six weeks) and long (12 months) term follow-up (Hafer, Becker et al. 2012). The outcome was the same in the Neo-PDGF trial that looked at high does of epoetin beta (Martinez, Kamar et al. 2010). Later trials similarly found

no evidence that erythropoietin improved function but also raised safety concerns (Aydin, Mallat et al. 2012). Similarly N-acetylcysteine did not improve DGF rates when given to over 100 deceased donors despite good suggestive data from animal research (Orban, Quintard et al. 2014).

4.2.2 *Other potential methods for HO-1 upregulation*

The time delay from dosing to upregulation of defensive mechanisms may limit the use of HA in acute ischaemic scenarios such as MI or stroke, but this makes it suitable for use in transplantation when delay is inevitable and strategic. However, HA is expensive and, as it can only be given intravenously, potentially difficult to give effectively. Despite this, it remains a better option than many of the harmful solutions used in preclinical studies but research continues into other options.

The same group that investigated the effect of hemin on healthy volunteers looked at other potential HO-1 upregulators. They gave healthy volunteers seven days of oral medications including various combinations of aspirin, statin and the sodium salt of R-alpha-lipoic acid to investigate if they could do the same job as expensive intravenous preparations (Bharucha, Choi et al. 2014). The group found that none of the medications had an effect on HO-1 protein concentration or venous monocyte HO-1 activity at two or seven day follow-up.

Despite evidence that curcumin induced HO-1 in human monocytes in culture (Hsu, Chu et al. 2008), healthy volunteer studies found that oral curcumin had low bioavailability and did not influence HO-1 mRNA or protein in PBMCs (Klickovic, Doberer et al. 2014).

IPC also upregulates HO-1 in animal renal tissue (Shokeir, Hussein et al. 2014) and the RCT currently recruiting to investigate the impact of RIPC on renal function after transplant should offer insights into protein expression.

4.2.3 *Treatment of deceased donors*

Despite its undeniable position as a life-changing opportunity to many ESRD patients, deceased donor transplantation remains challenging. Recipients of organs from unrelated living donors with poor HLA matching still have better graft function and survival compared to those who receive organs from well-matched deceased donors (Terasaki, Cecka et al. 1995).

Donor treatment remains a relatively underdeveloped area of clinical science, especially when one considers the substantial potential to intervene and improve organ function. It can be argued that given our knowledge of IRI, treatment of a potential organ donor offers the best chance to ameliorate the harmful downstream effects. Animal studies confirm that donor treatment prior to organ retrieval gives positive outcomes but attempts to transfer all but the most straightforward drugs to the clinical setting poses complex ethical issues (Kotsch, Ulrich et al. 2008). The recent publication of a randomised trial of French deceased donors treated with N-acetylecysteine may offer a model for future deceased donor research (Orban, Quintard et al. 2014). IPC appears to represent a lower-risk option and there are trials currently recruiting to investigate the effect of donor IPC on subsequent graft function (Feng 2010).

Pre-treatment of rat renal donors at onset of brain death with either steroids or P-selectin glycoprotein ligand (PSGL), a blocker of cell adhesion, improved recipient survival and reduced acute rejection episodes. It was hypothesised that it protected against IRI because there were fewer inflammatory markers in treated animals

(Pratschke, Kofla et al. 2001). Gasser et al found similar results after PSGL treatment and also showed improved function at day 200 (Gasser, Waaga et al. 2002). Reutzel-Selke et al expanded this concept and pre-treated rat donors with four immunosuppressive drugs commonly prescribed to human recipient treatment including steroid, MMF, tacrolimus and everolimus. They found that pre-treating with prednisolone or tacrolimus reduced the cytokine and cellular inflammatory response and as a result, significantly improved graft function and structure compared to control. MMF and everolimus also had benefits but did not reach significance (Reutzel-Selke, Zschockelt et al. 2003). Steroid treatment of donors is now routine after a randomised trial showed that it reduced inflammation in organs after transplant (Kotsch, Ulrich et al. 2008, Feng 2010).

Pre-treatment of organ donors with a variety of catecholamines reduced renal inflammatory cell infiltrates and improved renal function. The effect was maximal with dopamine (Gottmann, Brinkkoetter et al. 2006). When this was expanded to a randomised multi-centre clinical trial, dopamine pre-treatment reduced the need for recipient dialysis and has changed clinical practice (Schnuelle, Gottmann et al. 2009). The protective effect of dopamine is thought, in part, to be mediated through HO-1 induction (Schnuelle, Yard et al. 2004).

Donor pre-treatment with desmopressin is also associated with improved graft survival and this effect was enhanced when combined with dopamine (Benck, Gottmann et al. 2011). This is now widely used in donor management (McKeown, Bonser et al. 2012).

In pre-clinical studies, pre-treatment of brain dead donors with cobalt protoporphyrin led to an improvement in rat renal graft function and survival when compared to HO-1 blocker zinc protoporphyrin (Tullius, Nieminen-Kelha et al. 2002, Kotsch, Francuski et al. 2006). However the group concluded that while HO-1 induction in donors by pharmacological means was beneficial, there are limited compounds suitable for human use. HA may therefore offer a safe, effective alternative for preconditioning treatment of organ donors. However, a clinical trial of HA in donors is a long way off. While donor treatment with dopamine, desmopressin and steroids is now common, many molecular pathways of IRI including HO-1 remain unexplored and have not percolated through to human investigation (Feng 2010).

In their article Abt et al (Abt, Marsh et al. 2013) highlight the sparse number of donor intervention trials and discuss the ethical issues limiting clinical research. While they focus on the USA, many of their points are equally valid to the UK. Approval is difficult as, once a donor has been identified, the consent process no longer applies to only the donors but also to any family members, waiting-list candidates, multiple retrieval teams and organ recipients. By its very nature, clinical research also has the potential to cause harm and this risk may be counter to the wishes of the donor and family. The historical deceased donor trials used proxy consent but this does not address recipient consent, which poses further ethical issues. Given the complex nature of transplant, doctors treating the donor are usually some distance away and not linked to the multiple recipient care teams so that information about donor treatment cannot be quickly and clearly disseminated. These barriers mean that innovation is stifled and clinical trials for deceased donation are often abandoned reducing potential sources of knowledge.

4.2.4 *Treatment of organs*

As discussed in **4.2.3**, ethical and legal rules mean few interventions have been trialled in deceased donors. The same rules do not apply to isolated, retrieved organs and this offers a potential window of intervention.

One example of such an intervention is the development of machine perfusion. Machine perfusion has been shown to be beneficial to early kidney function and longer-term graft survival compared to conventional cold storage methods and is now in routine use (Moers, Smits et al. 2009).

A novel research focus is the development of organ perfusion fluids containing protective compounds including carbon monoxide (CO). A CO-containing solution reduced proinflammatory mediators post renal transplant and improved kidney function in an animal model. Similar biochemical changes were seen in human organs that were not fit for transplant and this should be expanded (Ozaki, Yoshida et al. 2012). However, there is reluctance to pursue this avenue because once the organ is in chilled isolation and not actively metabolising, it could be too late to impact on the preceding ischaemic inflammatory processes. This may explain why there are no further registered trials investigating organ treatment.

4.3 What next?

The HOT study used renal transplant as a model of IRI and it is hoped that these findings can be extrapolated to other pathologies where upregulation of HO-1 is known to be protective. The HOT study results have been used as preliminary data for a sample size calculation for a follow-on study in our unit to investigate the use of HA to prevent acute kidney injury after cardiac surgery.

4.3.1 *Expanding the HOT study*

The primary aim of the HOT study was to investigate the safety and efficacy of HO-1 induction by HA. Although there was a trend to protection against DGF, the study was not powered to this effect and therefore larger studies are required to see if upregulated HO-1 in PBMCs and renal tissue translates to the improved functional outcomes seen in animal work (Gueler, Park et al. 2007, Ferenbach, Nkejabega et al. 2011).

4.3.1.1 Plan for HOT2

The sample size calculation for HOT2 has suggested that in order to detect a difference in clinical outcome, namely DGF, 350 - 400 participants are required. In the HOT study, 82% of all recipients of single kidney, deceased donor transplants met eligibility criteria and of them, 83% consented to the trial. Last year, there were 100 renal transplants performed at Edinburgh Royal Infirmary (ERI) and therefore it would take at least 70 months to recruit 400 patients at ERI alone. One potential solution is to recruit from other centres too. The two nearest transplant units, Glasgow and Newcastle, have been approached and would be interested in principle.

The following points highlight the necessary amendments to the HOT protocol to allow effective expansion:

The primary outcome for HOT2 will be whether HA has an effect on the rate of DGF. This endpoint can be recorded retrospectively from online blood results. This will ensure that HOT2 is less invasive and demanding of researcher and participant.

The main hurdle to HOT expansion is manpower. In order to ensure that the HOT protocol was followed, the CI was responsible for all aspects of the seven-day study. The HOT study was labour intensive and it would be impossible to simply expand the same trial protocol to recruit 400 patients across more than one site. In the HOT study, the participant was approached, consented, randomised and given the HA infusion as soon as they arrived to ensure the recipient macrophages received maximum HA exposure prior to transplant. However, such a set-up would not be possible unless there was a designated Principle Investigator (PI) always on-call at each site to consent and give the HA.

One possible solution to recruitment would be to approach all eligible renal transplant waiting list patients with information about HOT2 at the start of the trial and they would sign a preliminary consent form if agreeable. This form would be kept in their notes to prevent unnecessary contact and effort if a recipient declined. The admitting doctor could reconfirm this on admission for transplant prior to randomisation. Once consent was confirmed, the doctor would also be responsible for randomisation, completing the paperwork, taking any preliminary bloods and organising the infusion.

It may be unreasonable to ask the admitting doctor to complete large volumes of paperwork in addition to their usual duties so, provided the initial eligibility questions had been recorded, another individual, such as a research nurse, could complete other paperwork in daytime hours.

A solution to randomisation would be to have a web based system that could be accessed by all the centres to allow different doctors to recruit and give infusions. This was too expensive for HOT but appropriate for HOT2 to ensure safe, transparent practice. It is probably also appropriate to have a web-based database for immediate data entry, which could be overseen by the designated researcher responsible for the trial. The designated person responsible for the trial must be a doctor but a specialist nurse could perform the day-to-day management and ERI has a successful track record of this process.

It is clear that HA upregulates HO-1 and therefore it will not be necessary for all HOT2 participants to have PBMC HO-1 protein and mRNA extracted and tested. If clarification of HO-1 upregulation is required, then a reduced, confirmatory analysis could be performed. HOT2 may require a lab-based, blinded technician to organise specimens, carry out the experiments and record the results but there is capacity at the local research institute for this.

It is not known whether dialysis removes HA from the circulation although after discussion with an expert, it was considered unlikely. However to be safe, HA was always given post dialysis. Unless this issue is clarified, HA will still need to be

given post dialysis and due to its potential toxicity to vessels, it must be given into a large vein. Therefore, one way to simplify the protocol is to give the infusion in theatre post-induction. This would ensure that HA was always given in a controlled manner into the central line, which is usually inserted by the anaesthetist at the start of the procedure.

The HOT study protocol gave two doses of HA and the licence for treatment of porphyria allows a maximum of four doses. Therefore, it may be appropriate to increase the dose of HA to investigate if DGF is dose-dependent. This may be limited by appropriate venous access but if the numbers of participants was sufficient, the study could subgroup patients into different dosing regimes.

The HOT2 protocol would not require a renal tissue biopsy. The biopsy was problematic to both participants and researchers and we do not consider the results to be worth the associated risks.

Summary

This MD concludes that HA is safe and effective in renal transplant recipients as reported in a phase II, randomised, placebo controlled, blinded, single-centre study. The primary outcome was achieved and demonstrated for the first time that HA induces HO-1 in peripheral and renal macrophages in kidney transplant recipients.

There was also evidence that HA increased HO-1 expression in renal tissue. There was no evidence that HA improved renal function or reduced injury as seen in animal models but it is recognised that the sample size was small and the study was not powered to these endpoints.

Larger studies are planned to determine the impact of HO-1 upregulation on clinical outcomes and evaluate the benefit to patients at risk of IRI. The plans for HOT2 are also expanded.

References

Abt, P. L., C. L. Marsh, T. B. Dunn, W. R. Hewitt, J. R. Rodrigue, J. M. Ham and S. Feng (2013). "Challenges to research and innovation to optimize deceased donor organ quality and quantity." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **13**(6): 1400-1404.

Adin, C. A., B. P. Croker and A. Agarwal (2005). "Protective effects of exogenous bilirubin on ischemia-reperfusion injury in the isolated, perfused rat kidney." American journal of physiology. Renal physiology **288**(4): F778-784.

Akagi, R., T. Takahashi and S. Sassa (2002). "Fundamental role of heme oxygenase in the protection against ischemic acute renal failure." Japanese journal of pharmacology **88**(2): 127-132.

Amador, A., L. Grande, J. Marti, R. Deulofeu, R. Miquel, A. Sola, G. Rodriguez-Laiz, J. Ferrer, C. Fondevila, R. Charco, J. Fuster, G. Hotter and J. C. Garcia-Valdecasas (2007). "Ischemic pre-conditioning in deceased donor liver transplantation: a prospective randomized clinical trial." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **7**(9): 2180-2189.

Amersi, F., R. Buelow, H. Kato, B. Ke, A. J. Coito, X. D. Shen, D. Zhao, J. Zaky, J. Melinek, C. R. Lassman, J. K. Kolls, J. Alam, T. Ritter, H. D. Volk, D. G. Farmer, R. M. Ghobrial, R. W. Busuttil and J. W. Kupiec-Weglinski (1999). "Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury." The Journal of clinical investigation **104**(11): 1631-1639.

Andersen, M. H., R. B. Sorensen, M. K. Brimnes, I. M. Svane, J. C. Becker and P. thor Straten (2009). "Identification of heme oxygenase-1-specific regulatory CD8+ T cells in cancer patients." The Journal of clinical investigation **119**(8): 2245-2256.

Anderson, J. L. and S. K. Chapman (2005). "Ligand probes for heme proteins." Dalton transactions (1): 13-24.

Araujo, J. A., M. Zhang and F. Yin (2012). "Heme oxygenase-1, oxidation, inflammation, and atherosclerosis." Frontiers in pharmacology **3**: 119.

Audard, V., M. Matignon, K. Dahan, P. Lang and P. Grimbert (2008). "Renal transplantation from extended criteria cadaveric donors: problems and perspectives overview." Transplant international : official journal of the European Society for Organ Transplantation **21**(1): 11-17.

Aydin, Z., M. J. Mallat, A. F. Schaapherder, A. J. van Zonneveld, C. van Kooten, T. J. Rabelink and J. W. de Fijter (2012). "Randomized trial of short-course high-dose erythropoietin in donation after cardiac death kidney transplant recipients."

American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **12**(7): 1793-1800.

Baan, C., A. Peeters, F. Lemos, A. Uitterlinden, I. Doxiadis, F. Claas, J. Ijzermans, J. Roodnat and W. Weimar (2004). "Fundamental role for HO-1 in the self-protection of renal allografts." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **4**(5): 811-818.

Balla, J., G. Balla, V. Jeney, G. Kakuk, H. S. Jacob and G. M. Vercellotti (2000). "Ferriporphyrins and endothelium: a 2-edged sword-promotion of oxidation and induction of cytoprotectants." Blood **95**(11): 3442-3450.

Balla, J., G. Balla, K. Nath and G. Vercellatti (1992). "Endothelial cell heme oxygenase and ferritin induction by heme proteins: a possible mechanism limiting shock damage." Clinical research **40**: 323A.

Balla, J., H. S. Jacob, G. Balla, K. Nath, J. W. Eaton and G. M. Vercellotti (1993). "Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage." Proceedings of the National Academy of Sciences of the United States of America **90**(20): 9285-9289.

Basile, D. P. (2007). "The endothelial cell in ischemic acute kidney injury: implications for acute and chronic function." Kidney international **72**(2): 151-156.

Belcher, J. D., H. Mahaseth, T. E. Welch, L. E. Otterbein, R. P. Hebbel and G. M. Vercellotti (2006). "Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice." The Journal of clinical investigation **116**(3): 808-816.

Benck, U., U. Gottmann, S. Hoeger, A. Lammert, D. Rose, D. Boesebeck, W. Lauchart, R. Birck, C. Weiss, B. K. Kramer, B. A. Yard and P. Schnuelle (2011). "Donor desmopressin is associated with superior graft survival after kidney transplantation." Transplantation **92**(11): 1252-1258.

Bharucha, A. E., K. M. Choi, J. J. Saw, S. J. Gibbons, G. F. Farrugia, D. A. Carlson and A. R. Zinsmeister (2014). "Effects of aspirin & simvastatin and aspirin, simvastatin, & lipoic acid on heme oxygenase-1 in healthy human subjects." Neurogastroenterology and motility **26**(10): 1437-1442.

Bharucha, A. E., A. Kulkarni, K. M. Choi, M. Camilleri, M. Lempke, G. J. Brunn, S. J. Gibbons, A. R. Zinsmeister and G. Farrugia (2010). "First-in-human study demonstrating pharmacological activation of heme oxygenase-1 in humans." Clinical pharmacology and therapeutics **87**(2): 187-190.

Blydt-Hansen, T. D., M. Katori, C. Lassman, B. Ke, A. J. Coito, S. Iyer, R. Buelow, R. Ettenger, R. W. Busuttil and J. W. Kupiec-Weglinski (2003). "Gene transfer-induced local heme oxygenase-1 overexpression protects rat kidney transplants from

ischemia/reperfusion injury." Journal of the American Society of Nephrology : JASN **14**(3): 745-754.

Bonventre, J. V. (2002). "Kidney ischemic preconditioning." Current opinion in nephrology and hypertension **11**(1): 43-48.

Bonventre, J. V. and L. Yang (2010). "Kidney injury molecule-1." Current opinion in critical care **16**(6): 556-561.

Boyle, J. J., M. Johns, J. Lo, A. Chiodini, N. Ambrose, P. C. Evans, J. C. Mason and D. O. Haskard (2011). "Heme induces heme oxygenase 1 via Nrf2: role in the homeostatic macrophage response to intraplaque hemorrhage." Arteriosclerosis, thrombosis, and vascular biology **31**(11): 2685-2691.

Brodsky, S. V., T. Yamamoto, T. Tada, B. Kim, J. Chen, F. Kajiya and M. S. Goligorsky (2002). "Endothelial dysfunction in ischemic acute renal failure: rescue by transplanted endothelial cells." American journal of physiology. Renal physiology **282**(6): F1140-1149.

Brouard, S., L. E. Otterbein, J. Anrather, E. Tobiasch, F. H. Bach, A. M. Choi and M. P. Soares (2000). "Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis." The Journal of Experimental Medicine **192**(7): 1015-1026.

Brune, B. and V. Ullrich (1987). "Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase." Molecular pharmacology **32**(4): 497-504.

Cao, Q., Y. Wang and D. C. Harris (2013). "Pathogenic and protective role of macrophages in kidney disease." American journal of physiology. Renal physiology **305**(1): F3-11.

Chen, Y., H. Zheng, X. Wang, Z. Zhou, A. Luo and Y. Tian (2013). "Remote ischemic preconditioning fails to improve early renal function of patients undergoing living-donor renal transplantation: a randomized controlled trial." Transplantation **95**(2): e4-6.

Chen, Y. H., S. J. Lin, M. W. Lin, H. L. Tsai, S. S. Kuo, J. W. Chen, M. J. Charng, T. C. Wu, L. C. Chen, Y. A. Ding, W. H. Pan, Y. S. Jou and L. Y. Chau (2002). "Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients." Human Genetics **111**(1): 1-8.

Coca, S. G. and C. R. Parikh (2008). "Urinary biomarkers for acute kidney injury: perspectives on translation." Clinical journal of the American Society of Nephrology : CJASN **3**(2): 481-490.

Cochrane, J., B. T. Williams, A. Banerjee, A. H. Harken, T. J. Burke, C. B. Cairns and J. I. Shapiro (1999). "Ischemic preconditioning attenuates functional, metabolic,

and morphologic injury from ischemic acute renal failure in the rat." Renal Failure **21**(2): 135-145.

Cohen, B., J. M. Smits, B. Haase, G. Persijn, Y. Vanrenterghem and U. Frei (2005). "Expanding the donor pool to increase renal transplantation." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **20**(1): 34-41.

Correa-Costa, M., H. Azevedo, M. T. Amano, G. M. Goncalves, M. I. Hyane, M. A. Cenedeze, P. G. Renesto, A. Pacheco-Silva, C. A. Moreira-Filho and N. O. Camara (2012). "Transcriptome analysis of renal ischemia/reperfusion injury and its modulation by ischemic pre-conditioning or hemin treatment." PloS one **7**(11): e49569.

Courtney, A. E. and A. P. Maxwell (2008). "Heme oxygenase 1: does it have a role in renal cytoprotection?" American journal of kidney diseases : the official journal of the National Kidney Foundation **51**(4): 678-690.

Courtney, A. E., P. T. McNamee, D. Middleton, S. Heggarty, C. C. Patterson and A. P. Maxwell (2007). "Association of functional heme oxygenase-1 gene promoter polymorphism with renal transplantation outcomes." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **7**(4): 908-913.

Day, Y. J., L. Huang, H. Ye, J. Linden and M. D. Okusa (2005). "Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages." American journal of physiology. Renal physiology **288**(4): F722-731.

de Almeida, M. C., A. C. Silva, A. Barral and M. Barral Netto (2000). "A simple method for human peripheral blood monocyte isolation." Memorias do Instituto Oswaldo Cruz **95**(2): 221-223.

Debout, A., Y. Foucher, K. Trebern-Launay, C. Legendre, H. Kreis, G. Mourad, V. Garrigue, E. Morelon, F. Buron, L. Rostaing, N. Kamar, M. Kessler, M. Ladriere, A. Poignas, A. Blidi, J. P. Soulillou, M. Giral and E. Dantan (2015). "Each additional hour of cold ischemia time significantly increases the risk of graft failure and mortality following renal transplantation." Kidney international **87**(2): 343-349.

Deshane, J., J. Kim, S. Bolisetty, T. D. Hock, N. Hill-Kapturczak and A. Agarwal (2010). "Sp1 regulates chromatin looping between an intronic enhancer and distal promoter of the human heme oxygenase-1 gene in renal cells." The Journal of biological chemistry **285**(22): 16476-16486.

Devarajan, P. (2006). "Update on mechanisms of ischemic acute kidney injury." Journal of the American Society of Nephrology : JASN **17**(6): 1503-1520.

Doberer, D., A. Haschemi, M. Andreas, T. C. Zapf, B. Clive, M. Jeitler, H. Heinzl, O. Wagner, M. Wolzt and M. Bilban (2010). "Haem arginate infusion stimulates

haem oxygenase-1 expression in healthy subjects." British journal of pharmacology **161**(8): 1751-1762.

Dore, S., M. Takahashi, C. D. Ferris, R. Zakhary, L. D. Hester, D. Guastella and S. H. Snyder (1999). "Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury." Proceedings of the National Academy of Sciences of the United States of America **96**(5): 2445-2450.

Doshi, M. D. and L. G. Hunsicker (2007). "Short- and long-term outcomes with the use of kidneys and livers donated after cardiac death." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **7**(1): 122-129.

Eltzschig, H. K. and C. D. Collard (2004). "Vascular ischaemia and reperfusion injury." British medical bulletin **70**: 71-86.

Erwig, L. P., D. C. Kluth and A. J. Rees (2001). "Macrophages in renal inflammation." Current opinion in nephrology and hypertension **10**(3): 341-347.

Exner, M., G. A. Bohmig, M. Schillinger, H. Regele, B. Watschinger, W. H. Horl, M. Raith, C. Mannhalter and O. F. Wagner (2004). "Donor heme oxygenase-1 genotype is associated with renal allograft function." Transplantation **77**(4): 538-542.

Exner, M., E. Minar, O. Wagner and M. Schillinger (2004). "The role of heme oxygenase-1 promoter polymorphisms in human disease." Free radical biology & medicine **37**(8): 1097-1104.

Farombi, E. O. and Y. J. Surh (2006). "Heme oxygenase-1 as a potential therapeutic target for hepatoprotection." Journal of biochemistry and molecular biology **39**(5): 479-491.

Fellstrom, B., H. Holdaas, A. G. Jardine, I. Holme, G. Nyberg, P. Fauchald, C. Gronhagen-Riska, S. Madsen, H. H. Neumayer, E. Cole, B. Maes, P. Ambuhl, A. G. Olsson, A. Hartmann, J. O. Logan, T. R. Pedersen and I. Assessment of Lescol in Renal Transplantation Study (2004). "Effect of fluvastatin on renal end points in the Assessment of Lescol in Renal Transplant (ALERT) trial." Kidney international **66**(4): 1549-1555.

Feng, S. (2010). "Donor intervention and organ preservation: where is the science and what are the obstacles?" American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **10**(5): 1155-1162.

Ferenbach, D. A., D. C. Kluth and J. Hughes (2010). "Hemeoxygenase-1 and renal ischaemia-reperfusion injury." Nephron. Experimental nephrology **115**(3): e33-37.

Ferenbach, D. A., N. C. Nkejabega, J. McKay, A. K. Choudhary, M. A. Vernon, M. F. Beesley, S. Clay, B. C. Conway, L. P. Marson, D. C. Kluth and J. Hughes (2011). "The induction of macrophage hemeoxygenase-1 is protective during acute kidney injury in aging mice." Kidney international **79**(9): 966-976.

Ferenbach, D. A., V. Ramdas, N. Spencer, L. Marson, I. Anegon, J. Hughes and D. C. Kluth (2010). "Macrophages expressing heme oxygenase-1 improve renal function in ischemia/reperfusion injury." Molecular therapy : the journal of the American Society of Gene Therapy **18**(9): 1706-1713.

Ferris, C. D., S. R. Jaffrey, A. Sawa, M. Takahashi, S. D. Brady, R. K. Barrow, S. A. Tysoe, H. Wolosker, D. E. Baranano, S. Dore, K. D. Poss and S. H. Snyder (1999). "Haem oxygenase-1 prevents cell death by regulating cellular iron." Nature cell biology **1**(3): 152-157.

Fiebiger, W., C. Mitterbauer and R. Oberbauer (2004). "Health-related quality of life outcomes after kidney transplantation." Health and quality of life outcomes **2**: 2.

Field, M., V. Dronavalli, P. Mistry, M. Drayson, A. Ready, M. Cobbold and N. Inston (2014). "Urinary biomarkers of acute kidney injury in deceased organ donors--kidney injury molecule-1 as an adjunct to predicting outcome." Clinical transplantation **28**(7): 808-815.

Floerchinger, B., R. Oberhuber and S. G. Tullius (2012). "Effects of brain death on organ quality and transplant outcome." Transplantation reviews (Orlando) **26**(2): 54-59.

Fraser, S. M., R. Rajasundaram, A. Aldouri, S. Farid, G. Morris-Stiff, R. Baker, C. G. Newstead, G. J. Toogood, K. V. Menon and N. Ahmad (2010). "Acceptable outcome after kidney transplantation using "expanded criteria donor" grafts." Transplantation **89**(1): 88-96.

Furuichi, K., J. L. Gao and P. M. Murphy (2006). "Chemokine receptor CX3CR1 regulates renal interstitial fibrosis after ischemia-reperfusion injury." The American journal of pathology **169**(2): 372-387.

Gasser, M., A. M. Waaga, J. E. Kist-Van Holthe, S. M. Lenhard, I. Laskowski, G. D. Shaw, W. W. Hancock and N. L. Tilney (2002). "Normalization of brain death-induced injury to rat renal allografts by recombinant soluble P-selectin glycoprotein ligand." Journal of the American Society of Nephrology : JASN **13**(7): 1937-1945.

Gaston, R. S., G. M. Danovitch, P. L. Adams, J. J. Wynn, R. M. Merion, M. H. Deierhoi, R. A. Metzger, J. M. Cecka, W. E. Harmon, A. B. Leichtman, A. Spital, E. Blumberg, C. A. Herzog, R. A. Wolfe, D. B. Tyan, J. Roberts, R. Rohrer, F. K. Port and F. L. Delmonico (2003). "The report of a national conference on the wait list for kidney transplantation." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **3**(7): 775-785.

Gottmann, U., P. T. Brinkkoetter, M. Bechtler, S. Hoeger, C. Karle, M. Schaub, P. Schnuelle, B. Yard, F. J. van der Woude and C. Braun (2006). "Effect of pre-treatment with catecholamines on cold preservation and ischemia/reperfusion-injury in rats." Kidney international **70**(2): 321-328.

Gueler, F., J. K. Park, S. Rong, T. Kirsch, C. Lindschau, W. Zheng, M. Elger, A. Fiebeler, D. Fliser, F. C. Luft and H. Haller (2007). "Statins attenuate ischemia-reperfusion injury by inducing heme oxygenase-1 in infiltrating macrophages." The American journal of pathology **170**(4): 1192-1199.

Hafer, C., T. Becker, J. T. Kielstein, E. Bahlmann, A. Schwarz, N. Grinzoff, D. Drzymala, I. Bonnard, N. Richter, F. Lehner, J. Klempnauer, H. Haller, J. Traeder and D. Fliser (2012). "High-dose erythropoietin has no effect on short- or long-term graft function following deceased donor kidney transplantation." Kidney international **81**(3): 314-320.

Hall, I. E., M. D. Doshi, P. P. Reese, R. J. Marcus, H. Thiessen-Philbrook and C. R. Parikh (2012). "Association between peritransplant kidney injury biomarkers and 1-year allograft outcomes." Clinical journal of the American Society of Nephrology : CJASN **7**(8): 1224-1233.

Hall, I. E., S. G. Yarlagadda, S. G. Coca, Z. Wang, M. Doshi, P. Devarajan, W. K. Han, R. J. Marcus and C. R. Parikh (2010). "IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation." Journal of the American Society of Nephrology : JASN **21**(1): 189-197.

Hammad, F. T., S. Al-Salam and L. Lubbad (2012). "Curcumin provides incomplete protection of the kidney in ischemia reperfusion injury." Physiological research **61**(5): 503-511.

Hayashi, S., R. Takamiya, T. Yamaguchi, K. Matsumoto, S. J. Tojo, T. Tamatani, M. Kitajima, N. Makino, Y. Ishimura and M. Suematsu (1999). "Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme." Circulation research **85**(8): 663-671.

Hill-Kapturczak, C., Agarwal. (2002). "Heme oxygenase and the kidney. ." DNA and cell biology **21**(4): 307-321.

Hill-Kapturczak, N., E. Sikorski, C. Voakes, J. Garcia, H. S. Nick and A. Agarwal (2003). "An internal enhancer regulates heme- and cadmium-mediated induction of human heme oxygenase-1." American journal of physiology. Renal physiology **285**(3): F515-523.

Hill-Kapturczak, N., V. Thamilselvan, F. Liu, H. S. Nick and A. Agarwal (2001). "Mechanism of heme oxygenase-1 gene induction by curcumin in human renal proximal tubule cells." American journal of physiology. Renal physiology **281**(5): F851-859.

Hock, T. D., K. Liby, M. M. Wright, S. McConnell, M. Schorpp-Kistner, T. M. Ryan and A. Agarwal (2007). "JunB and JunD regulate human heme oxygenase-1 gene expression in renal epithelial cells." The Journal of biological chemistry **282**(9): 6875-6886.

Hollmen, M. E., L. E. Kyllonen, K. A. Inkinen, M. L. Lalla and K. T. Salmela (2011). "Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation." Kidney international **79**(1): 89-98.

Holzen, J. P., C. August, R. Bahde, E. Minin, D. Lang, S. Heidenreich, K. H. Dietl and H. U. Spiegel (2008). "Influence of heme oxygenase-1 on microcirculation after kidney transplantation." The Journal of surgical research **148**(2): 126-135.

Hsu, H. Y., L. C. Chu, K. F. Hua and L. K. Chao (2008). "Heme oxygenase-1 mediates the anti-inflammatory effect of Curcumin within LPS-stimulated human monocytes." Journal of cellular physiology **215**(3): 603-612.

Huang, Y., J. Shan, C. Wang, J. Ma, D. Li, L. Li, S. Li and Y. Li (2009). "Can ischemic preconditioning alone really protect organs from ischemia reperfusion injury in transplantation." Transplant immunology **20**(3): 127-131.

Inguaggiato, P., L. Gonzalez-Michaca, A. J. Croatt, J. J. Haggard, J. Alam and K. A. Nath (2001). "Cellular overexpression of heme oxygenase-1 up-regulates p21 and confers resistance to apoptosis." Kidney international **60**(6): 2181-2191.

Ishii, T., K. Itoh, S. Takahashi, H. Sato, T. Yanagawa, Y. Katoh, S. Bannai and M. Yamamoto (2000). "Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages." The Journal of biological chemistry **275**(21): 16023-16029.

Israni, A. K., N. Li, B. B. Cizman, J. Snyder, J. Abrams, M. Joffe, T. Rebbeck and H. I. Feldman (2008). "Association of donor inflammation- and apoptosis-related genotypes and delayed allograft function after kidney transplantation." American journal of kidney diseases : the official journal of the National Kidney Foundation **52**(2): 331-339.

Jayaram, D., M. Kommareddi, R. S. Sung and F. L. Luan (2012). "Delayed graft function requiring more than one-time dialysis treatment is associated with inferior clinical outcomes." Clinical transplantation **26**(5): E536-543.

Jiang, X., W. Tian, Y. K. Sung, J. Qian and M. R. Nicolls (2014). "Macrophages in solid organ transplantation." Vascular cell **6**(1): 5.

Jo, S. K., S. A. Sung, W. Y. Cho, K. J. Go and H. K. Kim (2006). "Macrophages contribute to the initiation of ischaemic acute renal failure in rats." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **21**(5): 1231-1239.

Kainz, A., J. Wilflingseder, C. Mitterbauer, M. Haller, C. Burghuber, P. Perco, R. M. Langer, G. Heinze and R. Oberbauer (2010). "Steroid pretreatment of organ donors to prevent postischemic renal allograft failure: a randomized, controlled trial." Annals of internal medicine **153**(4): 222-230.

Katana, E., L. Skoura, D. Giakoustidis, D. Takoudas, N. Malisiovas and M. Daniilidis (2010). "Association between the heme oxygenase-1 promoter

polymorphism and renal transplantation outcome in Greece." Transplantation proceedings **42**(7): 2479-2485.

Katori, M., D. M. Anselmo, R. W. Busuttil and J. W. Kupiec-Weglinski (2002). "A novel strategy against ischemia and reperfusion injury: cytoprotection with heme oxygenase system." Transplant immunology **9**(2-4): 227-233.

Katori, M., R. Buelow, K. Bibo, J. Ma, A.J. Coito, S. Iyer, D. Southard, R. W. Busuttil and J. W. Kupiec-Weglinski (2002). "Heme oxygenase-1 overexpression potects rat hearts from cold ischaemia/reperfusion injury via an antiapoptotic pathway." Transplantation **73**(2): 287-292.

Kawashima, A., Y. Oda, A. Yachie, S. Koizumi and I. Nakanishi (2002). "Heme oxygenase-1 deficiency: the first autopsy case." Human pathology **33**(1): 125-130.

Kelly, K. J., W. W. Williams, Jr., R. B. Colvin, S. M. Meehan, T. A. Springer, J. C. Gutierrez-Ramos and J. V. Bonventre (1996). "Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury." The Journal of clinical investigation **97**(4): 1056-1063.

Kim, W. H., J. H. Lee, G. S. Kim, H. Y. Sim and S. J. Kim (2014). "The effect of remote ischemic postconditioning on graft function in patients undergoing living donor kidney transplantation." Transplantation **98**(5): 529-536.

Kinderlerer, A. R., I. Pombo Gregoire, S. S. Hamdulay, F. Ali, R. Steinberg, G. Silva, N. Ali, B. Wang, D. O. Haskard, M. P. Soares and J. C. Mason (2009). "Heme oxygenase-1 expression enhances vascular endothelial resistance to complement-mediated injury through induction of decay-accelerating factor: a role for increased bilirubin and ferritin." Blood **113**(7): 1598-1607.

Kinsey, G. R., L. Li and M. D. Okusa (2008). "Inflammation in acute kidney injury." Nephron. Experimental nephrology **109**(4): e102-107.

Kirkby, K. A. and C. A. Adin (2006). "Products of heme oxygenase and their potential therapeutic applications." American journal of physiology. Renal physiology **290**(3): F563-571.

Klickovic, U., D. Doberer, G. Gouya, S. Aschauer, S. Weisshaar, A. Storka, M. Bilban and M. Wolzt (2014). "Human pharmacokinetics of high dose oral curcumin and its effect on heme oxygenase-1 expression in healthy male subjects." BioMed research international **2014**: 458592.

Kluth, D. C., L. P. Erwig and A. J. Rees (2004). "Multiple facets of macrophages in renal injury." Kidney international **66**(2): 542-557.

Koneru, B., A. Fisher, Y. He, K. M. Klein, J. Skurnick, D. J. Wilson, A. N. de la Torre, A. Merchant, R. Arora and A. K. Samanta (2005). "Ischemic preconditioning in deceased donor liver transplantation: a prospective randomized clinical trial of safety and efficacy." Liver transplantation **11**(2): 196-202.

Koneru, B., A. Shareef, G. Dikdan, K. Desai, K. M. Klein, B. Peng, R. H. Wachsberg, A. N. de la Torre, M. Debroy, A. Fisher, D. J. Wilson and A. K. Samanta (2007). "The ischemic preconditioning paradox in deceased donor liver transplantation-evidence from a prospective randomized single blind clinical trial." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **7**(12): 2788-2796.

Koo, D. D. and S. Fuggle (2000). "Impact of Ischaemia/Reperfusion Injury and Early Inflammatory Responses in KIdney Transplantation." Transplantation Reviews **14**(4): 210-224.

Kotsch, K., M. Francuski, A. Pascher, R. Klemz, M. Seifert, J. Mittler, G. Schumacher, R. Buelow, H. D. Volk, S. G. Tullius, P. Neuhaus and J. Pratschke (2006). "Improved long-term graft survival after HO-1 induction in brain-dead donors." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **6**(3): 477-486.

Kotsch, K., F. Ulrich, A. Reutzel-Selke, A. Pascher, W. Faber, P. Warnick, S. Hoffman, M. Francuski, C. Kunert, O. Kuecuek, G. Schumacher, C. Wesslau, A. Lun, S. Kohler, S. Weiss, S. G. Tullius, P. Neuhaus and J. Pratschke (2008). "Methylprednisolone therapy in deceased donors reduces inflammation in the donor liver and improves outcome after liver transplantation: a prospective randomized controlled trial." Annals of surgery **248**(6): 1042-1050.

Kovtunovych, G., M. C. Ghosh, W. Ollivierre, R. P. Weitzel, M. A. Eckhaus, J. F. Tisdale, A. Yachie and T. A. Rouault (2014). "Wild-type macrophages reverse disease in heme oxygenase 1-deficient mice." Blood **124**(9): 1522-1530.

Land, W., H. Schneeberger, S. Schleibner, W. D. Illner, D. Abendroth, G. Rutili, K. E. Arfors and K. Messmer (1994). "The beneficial effect of human recombinant superoxide dismutase on acute and chronic rejection events in recipients of cadaveric renal transplants." Transplantation **57**(2): 211-217.

Lee, S., S. Huen, H. Nishio, S. Nishio, H. K. Lee, B. S. Choi, C. Ruhrberg and L. G. Cantley (2011). "Distinct macrophage phenotypes contribute to kidney injury and repair." Journal of the American Society of Nephrology : JASN **22**(2): 317-326.

Lemos, F. B., J. N. Ijzermans, P. E. Zondervan, A. M. Peeters, S. van den Engel, W. M. Mol, W. Weimar and C. C. Baan (2003). "Differential expression of heme oxygenase-1 and vascular endothelial growth factor in cadaveric and living donor kidneys after ischemia-reperfusion." Journal of the American Society of Nephrology : JASN **14**(12): 3278-3287.

Li, L. and M. D. Okusa (2010). "Macrophages, dendritic cells, and kidney ischemia-reperfusion injury." Seminars in nephrology **30**(3): 268-277.

Li, Q. F., Y. S. Zhu, H. Jiang, H. Xu and Y. Sun (2009). "Heme oxygenase-1 mediates the anti-inflammatory effect of isoflurane preconditioning in LPS-stimulated macrophages." Acta pharmacologica sinica **30**(2): 228-234.

Li, S. D., L. Wang, K. Y. Wang, P. Liang, G. Wu, K. Q. Zhang, Q. S. Li and F. S. Jin (2011). "Heme oxygenase-1 expression and its significance for acute rejection following kidney transplantation in rats." Transplantation proceedings **43**(5): 1980-1984.

Linden, I. B., O. Tokola, M. Karlsson and R. Tenhunen (1987). "Fate of haem after parenteral administration of haem arginate to rabbits." Journal of pharmacy and pharmacology **39**(2): 96-102.

Lindenblatt, N., R. Bordel, W. Schareck, M. D. Menger and B. Vollmar (2004). "Vascular heme oxygenase-1 induction suppresses microvascular thrombus formation in vivo." Arteriosclerosis, thrombosis, and vascular biology **24**(3): 601-606.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lu, X., J. Chen-Roetling and R. F. Regan (2014). "Systemic hemin therapy attenuates blood-brain barrier disruption after intracerebral hemorrhage." Neurobiology of disease **70**: 245-251.

Maines, M. D., R. D. Mayer, J. F. Ewing and W. K. McCoubrey, Jr. (1993). "Induction of kidney heme oxygenase-1 (HSP32) mRNA and protein by ischemia/reperfusion: possible role of heme as both promotor of tissue damage and regulator of HSP32." Journal of pharmacology and experimental therapeutics **264**(1): 457-462.

Malyszko, J., E. Koc-Zorawska, J. S. Malyszko and M. Mysliwiec (2010). "Kidney injury molecule-1 correlates with kidney function in renal allograft recipients." Transplantation proceedings **42**(10): 3957-3959.

Martinez, F., N. Kamar, N. Pallet, P. Lang, A. Durrbach, Y. Lebranchu, A. Adem, S. Barbier, E. Cassuto-Viguier, F. Glowaki, Y. Le Meur, L. Rostaing, C. Legendre, O. Hermine, G. Choukroun and P. S. I. Neo (2010). "High dose epoetin beta in the first weeks following renal transplantation and delayed graft function: Results of the Neo-PDGF Study." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **10**(7): 1695-1700.

Martins, P. N., A. Reuzel-Selke, A. Jurisch, K. Atrott, A. Pascher, J. Pratschke, R. Buelow, P. Neuhaus, H. D. Volk and S. G. Tullius (2005). "Induction of carbon monoxide in the donor reduces graft immunogenicity and chronic graft deterioration." Transplantation proceedings **37**(1): 379-381.

Matsumoto, M., Y. Makino, T. Tanaka, H. Tanaka, N. Ishizaka, E. Noiri, T. Fujita and M. Nangaku (2003). "Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats." Journal of the American Society of Nephrology : JASN **14**(7): 1825-1832.

McCafferty, K., S. Forbes, C. Thiemermann and M. M. Yaqoob (2014). "The challenge of translating ischemic conditioning from animal models to humans: the role of comorbidities." Disease models & mechanisms **7**(12): 1321-1333.

McDaid, J., K. Yamashita, A. Chora, R. Ollinger, T. B. Strom, X. C. Li, F. H. Bach and M. P. Soares (2005). "Heme oxygenase-1 modulates the allo-immune response by promoting activation-induced cell death of T cells." The FASEB journal : official publication of the Federation of American Societies for Experimental Biology **19**(3): 458-460.

McKeown, D. W., R. S. Bonser and J. A. Kellum (2012). "Management of the heartbeating brain-dead organ donor." British journal of anaesthesia **108 Suppl 1**: i96-107.

McNally, S. J., E. M. Harrison, J. A. Ross, O. J. Garden and S. J. Wigmore (2006). "Curcumin induces heme oxygenase-1 in hepatocytes and is protective in simulated cold preservation and warm reperfusion injury." Transplantation **81**(4): 623-626.

Mense, S. M. and L. Zhang (2006). "Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases." Cell research **16**(8): 681-692.

Mishra, J., C. Dent, R. Tarabishi, M. M. Mitsnefes, Q. Ma, C. Kelly, S. M. Ruff, K. Zahedi, M. Shao, J. Bean, K. Mori, J. Barasch and P. Devarajan (2005). "Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery." Lancet **365**(9466): 1231-1238.

Mishra, J., Q. Ma, C. Kelly, M. Mitsnefes, K. Mori, J. Barasch and P. Devarajan (2006). "Kidney NGAL is a novel early marker of acute injury following transplantation." Paediatric Nephrology **21** (6): 856-863.

Mishra, J., K. Mori, Q. Ma, C. Kelly, J. Yang, M. Mitsnefes, J. Barasch and P. Devarajan (2004). "Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin." Journal of the American Society of Nephrology : JASN **15**(12): 3073-3082.

Moers, C., J. M. Smits, M. H. Maathuis, J. Treckmann, F. van Gelder, B. P. Napieralski, M. van Kasterop-Kutz, J. J. van der Heide, J. P. Squifflet, E. van Heurn, G. R. Kirste, A. Rahmel, H. G. Leuvenink, A. Paul, J. Pirenne and R. J. Ploeg (2009). "Machine perfusion or cold storage in deceased-donor kidney transplantation." The New England journal of medicine **360**(1): 7-19.

Moore, J., S. Shabir, S. Chand, A. Bentall, A. McClean, W. Chan, S. Jham, D. Benavente, A. Sharif, S. Ball, P. Cockwell and R. Borrows (2010). "Assessing and

comparing rival definitions of delayed renal allograft function for predicting subsequent graft failure." Transplantation **90**(10): 1113-1116.

Mori, K., H. T. Lee, D. Rapoport, I. R. Drexler, K. Foster, J. Yang, K. M. Schmidt-Ott, X. Chen, J. Y. Li, S. Weiss, J. Mishra, F. H. Cheema, G. Markowitz, T. Suganami, K. Sawai, M. Mukoyama, C. Kunis, V. D'Agati, P. Devarajan and J. Barasch (2005). "Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury." The Journal of clinical investigation **115**(3): 610-621.

Morimoto, K., K. Ohta, A. Yachie, Y. Yang, M. Shimizu, C. Goto, T. Toma, Y. Kasahara, H. Yokoyama, T. Miyata, H. Seki and S. Koizumi (2001). "Cytoprotective role of heme oxygenase (HO)-1 in human kidney with various renal diseases." Kidney international **60**(5): 1858-1866.

Murry, C. E., R. B. Jennings and K. A. Reimer (1986). "Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium." Circulation **74**(5): 1124-1136.

Nakagami, T., K. Toyomura, T. Kinoshita and S. Morisawa (1993). "A beneficial role of bile pigments as an endogenous tissue protector: anti-complement effects of biliverdin and conjugated bilirubin." Biochimica et biophysica acta **1158**(2): 189-193.

Nakao, A., J. S. Neto, S. Kanno, D. B. Stolz, K. Kimizuka, F. Liu, F. H. Bach, T. R. Billiar, A. M. Choi, L. E. Otterbein and N. Murase (2005). "Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **5**(2): 282-291.

Nath, K. A. (2006). "Heme oxygenase-1: a provenance for cytoprotective pathways in the kidney and other tissues." Kidney international **70**(3): 432-443.

Nath, K. A., Balla G, Vercellotti. (1992). "Induction of heme-oxygenase is a rapid, protective response in rhabdomyolysis in the rat. ." Journal of Clinical Investigation **90**: 267-270.

Nath, K. A., J. J. Haggard, A. J. Croatt, J. P. Grande, K. D. Poss and J. Alam (2000). "The indispensability of heme oxygenase-1 in protecting against acute heme protein-induced toxicity in vivo." The American journal of pathology **156**(5): 1527-1535.

Nath, K. A., G. M. Vercellotti, J. P. Grande, H. Miyoshi, C. V. Paya, J. C. Manivel, J. J. Haggard, A. J. Croatt, W. D. Payne and J. Alam (2001). "Heme protein-induced chronic renal inflammation: suppressive effect of induced heme oxygenase-1." Kidney international **59**(1): 106-117.

Neto, J. S., A. Nakao, K. Kimizuka, A. J. Romanosky, D. B. Stolz, T. Uchiyama, M. A. Nalesnik, L. E. Otterbein and N. Murase (2004). "Protection of transplant-induced

renal ischemia-reperfusion injury with carbon monoxide." American journal of physiology. Renal physiology **287**(5): F979-989.

Nijboer, W. N., T. A. Schuurs, J. A. van der Hoeven, S. Fekken, J. Wiersema-Buist, H. G. Leuvenink, S. Hofker, J. J. Homan van der Heide, W. J. van Son and R. J. Ploeg (2004). "Effect of brain death on gene expression and tissue activation in human donor kidneys." Transplantation **78**(7): 978-986.

Ogawa, K., J. Sun, S. Taketani, O. Nakajima, C. Nishitani, S. Sassa, N. Hayashi, M. Yamamoto, S. Shibahara, H. Fujita and K. Igarashi (2001). "Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1." The EMBO journal **20**(11): 2835-2843.

Ojo, A. O., J. A. Hanson, H. Meier-Kriesche, C. N. Okechukwu, R. A. Wolfe, A. B. Leichtman, L. Y. Agodoa, B. Kaplan and F. K. Port (2001). "Survival in recipients of marginal cadaveric donor kidneys compared with other recipients and wait-listed transplant candidates." Journal of the American Society of Nephrology : JASN **12**(3): 589-597.

Ollinger, R., P. Kogler, M. Biebl, M. Sieb, R. Sucher, C. Bosmuller, J. Troppmair, W. Mark, H. Weiss and R. Margreiter (2008). "Protein levels of heme oxygenase-1 during reperfusion in human kidney transplants with delayed graft function." Clinical transplantation **22**(4): 418-423.

Oniscu, G. C., H. Brown and J. L. Forsythe (2005). "Impact of cadaveric renal transplantation on survival in patients listed for transplantation." Journal of the American Society of Nephrology : JASN **16**(6): 1859-1865.

Orban, J. C., H. Quintard, E. Cassuto, P. Jambou, C. Samat-Long and C. Ichai (2014). "Effect of N-Acetylcysteine Pretreatment of Deceased Organ Donors on Renal Allograft Function: A Randomized Controlled Trial." Transplantation.

Otterbein, L. E., F. H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R. J. Davis, R. A. Flavell and A. M. Choi (2000). "Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway." Nature medicine **6**(4): 422-428.

Ozaki, K. S., J. Yoshida, S. Ueki, G. L. Pettigrew, N. Ghonem, R. M. Sico, L. Y. Lee, R. Shapiro, F. G. Lakkis, A. Pacheco-Silva and N. Murase (2012). "Carbon monoxide inhibits apoptosis during cold storage and protects kidney grafts donated after cardiac death." Transplant international : official journal of the European Society for Organ Transplantation **25**(1): 107-117.

Pae, H. O., G. S. Oh, B. M. Choi, S. C. Chae, Y. M. Kim, K. R. Chung and H. T. Chung (2004). "Carbon monoxide produced by heme oxygenase-1 suppresses T cell proliferation via inhibition of IL-2 production." Journal of immunology **172**(8): 4744-4751.

Paine, A., B. Eiz-Vesper, R. Blasczyk and S. Immenschuh (2010). "Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential." Biochemical pharmacology **80**(12): 1895-1903.

Parikh, C. R., A. Jani, J. Mishra, Q. Ma, C. Kelly, J. Barasch, C. L. Edelstein and P. Devarajan (2006). "Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **6**(7): 1639-1645.

Peyton, K. J., S. V. Reyna, G. B. Chapman, D. Ensenat, X. M. Liu, H. Wang, A. I. Schafer and W. Durante (2002). "Heme oxygenase-1-derived carbon monoxide is an autocrine inhibitor of vascular smooth muscle cell growth." Blood **99**(12): 4443-4448.

Pianta, T. J., P. W. Peake, J. W. Pickering, M. Kelleher, N. A. Buckley and Z. H. Endre (2014). "Clusterin in Kidney Transplantation: Novel Biomarkers Versus Serum Creatinine for Early Prediction of Delayed Graft Function." Transplantation.

Pimstone, N. R., P. Engel, R. Tenhunen, P. T. Seitz, H. S. Marver and R. Schmid (1971). "Inducible heme oxygenase in the kidney: a model for the homeostatic control of hemoglobin catabolism." The Journal of clinical investigation **50**(10): 2042-2050.

Poss, K. D. and S. Tonegawa (1997). "Reduced stress defense in heme oxygenase 1-deficient cells." Proceedings of the National Academy of Sciences of the United States of America **94**(20): 10925-10930.

Pratschke, J., G. Kofla, M. J. Wilhelm, A. Vergopoulos, I. Laskowski, G. D. Shaw, S. G. Tullius, H. D. Volk, P. Neuhaus and N. L. Tilney (2001). "Improvements in early behavior of rat kidney allografts after treatment of the brain-dead donor." Annals of surgery **234**(6): 732-740.

Pratschke, J., M. J. Wilhelm, I. Laskowski, M. Kusaka, F. Beato, S. G. Tullius, P. Neuhaus, W. W. Hancock and N. L. Tilney (2001). "Influence of donor brain death on chronic rejection of renal transplants in rats." Journal of the American Society of Nephrology : JASN **12**(11): 2474-2481.

Quiroga, I., P. McShane, D. D. Koo, D. Gray, P. J. Friend, S. Fuggle and C. Darby (2006). "Major effects of delayed graft function and cold ischaemia time on renal allograft survival." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **21**(6): 1689-1696.

Raval, C. M., J. L. Zhong, S. A. Mitchell and R. M. Tyrrell (2012). "The role of Bach1 in ultraviolet A-mediated human heme oxygenase 1 regulation in human skin fibroblasts." Free radical biology & medicine **52**(1): 227-236.

Redaelli, C. A., M. Wagner, C. Kulli, Y. H. Tian, D. Kubulus, L. Mazzucchelli, A. C. Wagner and M. K. Schilling (2001). "Hyperthermia-induced HSP expression correlates with improved rat renal isograft viability and survival in kidneys harvested from non-heart-beating donors." Transplant international : official journal of the European Society for Organ Transplantation **14**(6): 351-360.

Regan, R. F., Y. Guo and N. Kumar (2000). "Heme oxygenase-1 induction protects murine cortical astrocytes from hemoglobin toxicity." Neuroscience letters **282**(1-2): 1-4.

Reutzel-Selke, A., T. Zschockelt, C. Denecke, U. Bachmann, A. Jurisch, J. Pratschke, G. Schmidbauer, H. D. Volk, P. Neuhaus and S. G. Tullius (2003). "Short-term immunosuppressive treatment of the donor ameliorates consequences of ischemia/ reperfusion injury and long-term graft function in renal allografts from older donors." Transplantation **75**(11): 1786-1792.

Richards, J. A., S. J. Wigmore and L. R. Devey (2010). "Heme oxygenase system in hepatic ischemia-reperfusion injury." World journal of gastroenterology : WJG **16**(48): 6068-6078.

Ricardo, S. D., H. van Goor and A. E. Eddy (2008). "Macrophage diversity in renal injury and repair." The journal of clinical investigation **118**(11): 3522-3530

Riedl, M. A., A. Saxon and D. Diaz-Sanchez (2009). "Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway." Clinical immunology **130**(3): 244-251.

Roach, J. P., E. E. Moore, D. A. Partrick, S. S. Damle, C. C. Silliman, R. C. McIntyre, Jr. and A. Banerjee (2009). "Heme oxygenase-1 induction in macrophages by a hemoglobin-based oxygen carrier reduces endotoxin-stimulated cytokine secretion." Shock **31**(3): 251-257.

Ronco, C., M. Legrand, S. L. Goldstein, M. Hur, N. Tran, E. C. Howell, V. Cantaluppi, D. N. Cruz, K. Damman, S. M. Bagshaw, S. Di Somma and A. Lewington (2014). "Neutrophil gelatinase-associated lipocalin: ready for routine clinical use? An international perspective." Blood purification **37**(4): 271-285.

Rushworth, S. A., S. Shah and D. J. MacEwan (2011). "TNF mediates the sustained activation of Nrf2 in human monocytes." Journal of immunology **187**(2): 702-707.

Salahudeen, A. A., J. K. Jenkins, H. Huang, K. Ndebele and A. K. Salahudeen (2001). "Overexpression of heme oxygenase protects renal tubular cells against cold storage injury: studies using hemin induction and HO-1 gene transfer." Transplantation **72**(9): 1498-1504.

Schnuelle, P., U. Gottmann, S. Hoeger, D. Boesebeck, W. Lauchart, C. Weiss, M. Fischereder, K. W. Jauch, U. Heemann, M. Zeier, C. Hugo, P. Pisarski, B. K. Kramer, K. Lopau, A. Rahmel, U. Benck, R. Birck and B. A. Yard (2009). "Effects of donor pretreatment with dopamine on graft function after kidney transplantation: a

randomized controlled trial." JAMA: The journal of the American Medical Association **302**(10): 1067-1075.

Schnuelle, P., B. A. Yard, C. Braun, E. Dominguez-Fernandez, M. Schaub, R. Birck, J. Sturm, S. Post and F. J. van der Woude (2004). "Impact of donor dopamine on immediate graft function after kidney transplantation." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **4**(3): 419-426.

Selzner, N., M. Boehnert and M. Selzner (2012). "Preconditioning, postconditioning, and remote conditioning in solid organ transplantation: basic mechanisms and translational applications." Transplantation reviews (Orlando) **26**(2): 115-124.

Serur, D., S. Saal, J. Wang, J. Sullivan, R. Bologa, C. Hartono, D. Dadhania, J. Lee, L. M. Gerber, M. Goldstein, S. Kapur, W. Stubenbord, R. Belenkaya, M. Marin, S. Seshan, Q. Ni, D. Levine, T. Parker, K. Stenzel, B. Smith, R. Riggio and J. Cheigh (2011). "Deceased-donor kidney transplantation: improvement in long-term survival." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **26**(1): 317-324.

Sharif, A. and R. Borrows (2013). "Delayed graft function after kidney transplantation: the clinical perspective." American journal of kidney diseases : the official journal of the National Kidney Foundation **62**(1): 150-158.

Shibahara, S., M. Sato, R. M. Muller and T. Yoshida (1989). "Structural organization of the human heme oxygenase gene and the function of its promoter." European journal of biochemistry **179**(3): 557-563.

Shimizu, H., T. Takahashi, T. Suzuki, A. Yamasaki, T. Fujiwara, Y. Odaka, M. Hirakawa, H. Fujita and R. Akagi (2000). "Protective effect of heme oxygenase induction in ischemic acute renal failure." Critical care medicine **28**(3): 809-817.

Shiraishi, F., L. M. Curtis, L. Truong, K. Poss, G. A. Visner, K. Madsen, H. S. Nick and A. Agarwal (2000). "Heme oxygenase-1 gene ablation or expression modulates cisplatin-induced renal tubular apoptosis." American journal of physiology. Renal physiology **278**(5): F726-736.

Shokeir, A. A., A. M. Hussein, N. Barakat, A. Abdelaziz, M. Elgarba and A. Awadalla (2014). "Activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and Nrf-2-dependent genes by ischaemic pre-conditioning and post-conditioning: new adaptive endogenous protective responses against renal ischaemia/reperfusion injury." Acta physiologica **210**(2): 342-353.

Shoskes, D., C. Lapierre, M. Cruz-Correa, N. Muruve, R. Rosario, B. Fromkin, M. Braun and J. Copley (2005). "Beneficial effects of the bioflavonoids curcumin and quercetin on early function in cadaveric renal transplantation: a randomized placebo controlled trial." Transplantation **80**(11): 1556-1559.

Siedlecki, A., W. Irish and D. C. Brennan (2011). "Delayed graft function in the kidney transplant." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **11**(11): 2279-2296.

Sikorski, E. M., T. Hock, N. Hill-Kapturczak and A. Agarwal (2004). "The story so far: Molecular regulation of the heme oxygenase-1 gene in renal injury." American journal of physiology. Renal physiology **286**(3): F425-441.

Snoeijs, M. G., D. E. Schaubel, R. Hene, A. J. Hoitsma, M. M. Idu, J. N. Ijzermans, R. J. Ploeg, J. Ringers, M. H. Christiaans, W. A. Buurman and L. W. van Heurn (2010). "Kidneys from donors after cardiac death provide survival benefit." Journal of the American Society of Nephrology : JASN **21**(6): 1015-1021.

Soares, M. P., Y. Lin, J. Anrather, E. Csizmadia, K. Takigami, K. Sato, S. T. Grey, R. B. Colvin, A. M. Choi, K. D. Poss and F. H. Bach (1998). "Expression of heme oxygenase-1 can determine cardiac xenograft survival." Nature medicine **4**(9): 1073-1077.

Soares, M. P., M. P. Seldon, I. P. Gregoire, T. Vassilevskaia, P. O. Berberat, J. Yu, T. Y. Tsui and F. H. Bach (2004). "Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation." Journal of immunology **172**(6): 3553-3563.

Song, H., S. Hoeger, J. L. Hillebrands, I. Mandel, R. Loesel, G. Beck, L. Schilling, P. Schnuelle and B. Yard (2010). "CORMs protect endothelial cells during cold preservation, resulting in inhibition of intimal hyperplasia after aorta transplantation in rats." Transplant international : official journal of the European Society for Organ Transplantation **23**(11): 1144-1153.

Stocker, R., Y. Yamamoto, A. F. McDonagh, A. N. Glazer and B. N. Ames (1987). "Bilirubin is an antioxidant of possible physiological importance." Science **235**(4792): 1043-1046.

Sureshkumar, K. K., S. M. Hussain, T. Y. Ko, N. L. Thai and R. J. Marcus (2012). "Effect of high-dose erythropoietin on graft function after kidney transplantation: a randomized, double-blind clinical trial." Clinical journal of the American Society of Nephrology : CJASN **7**(9): 1498-1506.

Suttner, D. M. and P. A. Dennery (1999). "Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron." The FASEB journal : official publication of the Federation of American Societies for Experimental Biology **13**(13): 1800-1809.

Szeto, C. C., B. C. Kwan, K. B. Lai, F. M. Lai, K. M. Chow, G. Wang, C. C. Luk and P. K. Li (2010). "Urinary expression of kidney injury markers in renal transplant recipients." Clinical journal of the American Society of Nephrology : CJASN **5**(12): 2329-2337.

Szwarc, I., S. Soullier, N. Gayrard, C. Mejean, G. Mourad and A. Argiles (2007). "Ischemic postconditioning prevents ischemic acute renal failure." Transplantation proceedings **39**(8): 2554-2556.

Tenhunen, R., O. Tokola and I. B. Linden (1987). "Haem arginate: a new stable haem compound." Journal of pharmacy and pharmacology **39**(10): 780-786.

Terasaki, P. I., J. M. Cecka, D. W. Gjertson and S. Takemoto (1995). "High survival rates of kidney transplants from spousal and living unrelated donors." The New England journal of medicine **333**(6): 333-336.

Thorup, C., C. L. Jones, S. S. Gross, L. C. Moore and M. S. Goligorsky (1999). "Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS." The American journal of physiology **277**(6 Pt 2): F882-889.

Toda, N., T. Takahashi, S. Mizobuchi, H. Fujii, K. Nakahira, S. Takahashi, M. Yamashita, K. Morita, M. Hirakawa and R. Akagi (2002). "Tin chloride pretreatment prevents renal injury in rats with ischemic acute renal failure." Critical care medicine **30**(7): 1512-1522.

Traylor, A., T. Hock and N. Hill-Kapturczak (2007). "Specificity protein 1 and Smad-dependent regulation of human heme oxygenase-1 gene by transforming growth factor-beta1 in renal epithelial cells." American journal of physiology. Renal physiology **293**(3): F885-894.

Tsuchihashi, S., M. Livhits, Y. Zhai, R. W. Busuttil, J. A. Araujo and J. W. Kupiec-Weglinski (2006). "Basal rather than induced heme oxygenase-1 levels are crucial in the antioxidant cytoprotection." Journal of immunology **177**(7): 4749-4757.

Tullius, S. G., M. Nieminen-Kelha, R. Buelow, A. Reutzel-Selke, P. N. Martins, J. Pratschke, U. Bachmann, M. Lehmann, D. Southard, S. Iyer, G. Schmidbauer, B. Sawitzki, P. Reinke, P. Neuhaus and H. D. Volk (2002). "Inhibition of ischemia/reperfusion injury and chronic graft deterioration by a single-donor treatment with cobalt-protoporphyrin for the induction of heme oxygenase-1." Transplantation **74**(5): 591-598.

Tullius, S. G., A. Reutzel-Selke, F. Egermann, M. Nieminen-Kelha, S. Jonas, W. O. Bechstein, H. D. Volk and P. Neuhaus (2000). "Contribution of prolonged ischemia and donor age to chronic renal allograft dysfunction." Journal of the American Society of Nephrology : JASN **11**(7): 1317-1324.

Turpeinen, H., L. E. Kyllonen, J. Parkkinen, J. Laine, K. T. Salmela and J. Partanen (2007). "Heme oxygenase 1 gene polymorphisms and outcome of renal transplantation." International journal of immunogenetics **34**(4): 253-257.

van Timmeren, M. M., V. S. Vaidya, R. M. van Ree, L. H. Oterdoom, A. P. de Vries, R. O. Gans, H. van Goor, C. A. Stegeman, J. V. Bonventre and S. J. Bakker (2007). "High urinary excretion of kidney injury molecule-1 is an independent predictor of graft loss in renal transplant recipients." Transplantation **84**(12): 1625-1630.

Varadarajan, R., L. Golden-Mason, L. Young, P. McLoughlin, N. Nolan, G. McEntee, O. Traynor, J. Geoghegan, J. E. Hegarty and C. O'Farrelly (2004). "Nitric oxide in early ischaemia reperfusion injury during human orthotopic liver transplantation." Transplantation **78**(2): 250-256.

Wagener, F. A., H. E. van Beurden, J. W. von den Hoff, G. J. Adema and C. G. Figdor (2003). "The heme-heme oxygenase system: a molecular switch in wound healing." Blood **102**(2): 521-528.

Wagner, M., P. Cadetg, R. Ruf, L. Mazzucchelli, P. Ferrari and C. A. Redaelli (2003). "Heme oxygenase-1 attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts." Kidney international **63**(4): 1564-1573.

Wegiel, B., A. Hedblom, M. Li, D. Gallo, E. Csizmadia, C. Harris, Z. Nemeth, B. S. Zuckerbraun, M. Soares, J. L. Persson and L. E. Otterbein (2014). "Heme oxygenase-1 derived carbon monoxide permits maturation of myeloid cells." Cell death & disease **5**: e1139.

Weis, N., A. Weigert, A. von Knethen and B. Brune (2009). "Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants." Molecular biology of the cell **20**(5): 1280-1288.

Wever, K. E., R. Masereeuw, F. A. Wagener, V. G. Verweij, J. G. Peters, J. C. Pertijs, J. A. Van der Vliet, M. C. Warle and G. A. Rongen (2013). "Humoral signalling compounds in remote ischaemic preconditioning of the kidney, a role for the opioid receptor." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **28**(7): 1721-1732.

Wolfe, R. A., V. B. Ashby, E. L. Milford, A. O. Ojo, R. E. Ettenger, L. Y. Agodoa, P. J. Held and F. K. Port (1999). "Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant." The New England journal of medicine **341**(23): 1725-1730.

Wu, J., X. Feng, H. Huang, Z. Shou, X. Zhang, R. Wang, Y. Chen and J. Chen (2014). "Remote ischemic conditioning enhanced the early recovery of renal function in recipients after kidney transplantation: a randomized controlled trial." The Journal of surgical research **188**(1): 303-308.

Yachie, A., Y. Niida, T. Wada, N. Igarashi, H. Kaneda, T. Toma, K. Ohta, Y. Kasahara and S. Koizumi (1999). "Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency." The Journal of clinical investigation **103**(1): 129-135.

Yachie, A., T. Toma, K. Mizuno, H. Okamoto, S. Shimura, K. Ohta, Y. Kasahara and S. Koizumi (2003). "Heme oxygenase-1 production by peripheral blood monocytes during acute inflammatory illnesses of children." Experimental biology and medicine **228**(5): 550-556.

Yarlagadda, S. G., S. G. Coca, R. N. Formica, Jr., E. D. Poggio and C. R. Parikh (2009). "Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **24**(3): 1039-1047.

Yarlagadda, S. G., S. G. Coca, A. X. Garg, M. Doshi, E. Poggio, R. J. Marcus and C. R. Parikh (2008). "Marked variation in the definition and diagnosis of delayed graft function: a systematic review." Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **23**(9): 2995-3003.

Yoneya, R., Y. Nagashima, K. Sakaki, K. Hagiwara, H. Teraoka, H. Ozasa and S. Horikawa (2002). "Hemolysate pretreatment ameliorates ischemic acute renal injury in rats." Nephron **92**(2): 407-413.

Zhang, J., T. Ohta, A. Maruyama, T. Hosoya, K. Nishikawa, J. M. Maher, S. Shibahara, K. Itoh and M. Yamamoto (2006). "BRG1 interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative stress." Molecular and cellular biology **26**(21): 7942-7952.